[54] **PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS**

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[57] **ABSTRACT**

The present invention relates to processes for inserting DNA into eucaryotic cells, particularly DNA which includes a gene or genes coding for desired proteinaceous materials for which no selective criteria exist. The insertion of such DNA molecules is accomplished by cotransforming eucaryotic cells with such DNA together with a second DNA which corresponds to a gene coding for a selectable marker.

The invention further relates to processes for inserting into eucaryotic cells a multiplicity of DNA molecules including genes coding for desired proteinaceous materials by cotransformation with the desired genes and with amplifiable genes for a dominant selectable marker in the presence of successively higher amounts of an inhibitor. Alternatively, the insertion of multiple copies of desired genes is accomplished by transformation using DNA molecules formed by ligating a DNA molecule including the desired gene to a DNA molecule which includes an amplifiable gene coding for a dominant selectable phenotype such as a gene associated with resistance to a drug in the presence of successively higher amounts of an agent such as a drug against which the gene confers resistance so that only those eucaryotic cells into which multiple copies of the amplifiable gene have been inserted survive.

73 Claims, 2 Drawing Figures

#17 attach

COTRANSFORMATION OF EUCARYOTIC CELLS

DNA I CODING FOR DESIRED PROTEINACEOUS MATERIAL

DNA II CODING FOR SELECTABLE MARKER (tk+)

+

NUCLEUS
CHROMOSONAL DNA
EUCARYOTIC CELL (tk-)
CYTOPLASM
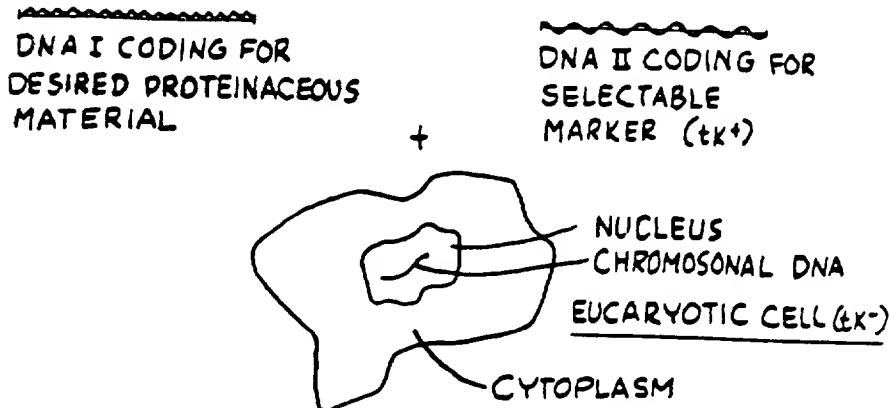
COTRANSFORM IN PRESENCE OF SELECTIVE CRITERIA (HAT)

Fig. 1.

EUCARYOTIC CELL (tk+)
DNA II
DNA I
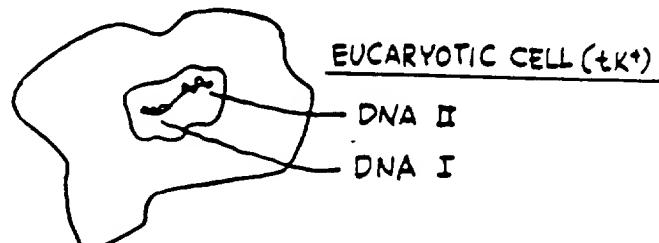
CULTURE IN PRESENCE OF SELECTIVE CRITERIA (HAT)

DESIRED MATERIAL

DESIRED MATERIAL

RECOVERY

DESIRED MATERIAL
(e.g. INTERFERON, INSULIN, ETC.)
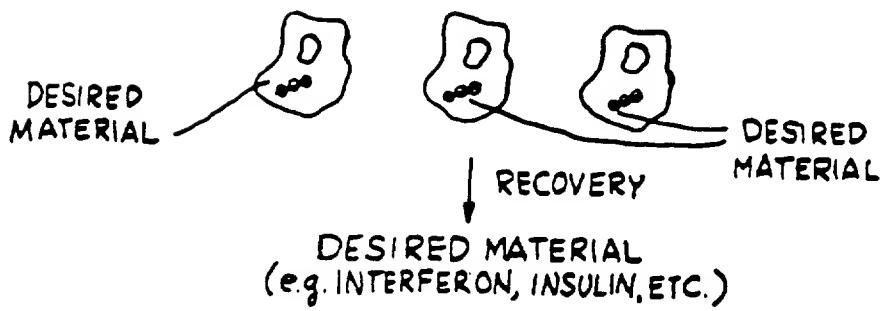
RESCUE OF pBR FROM TRANSFORMED MOUSE CELLS

Tet'   ■   Amp'
                                    O

HIND Ⅲ CLEAVED pBR 322

↓ COTRANSFORM MOUSE CELLS

Xba I   ■   Amp'   Xba I
                         O

pBR 322 INTEGRATED IN MOUSE DNA

↓ CLEAVE WITH Xba I
CIRCULARIZE WITH LIGASE
TRANSFORM E.COLI X 1776

Fig. 2.

MOUSE DNA INSERT

Amp'

● O
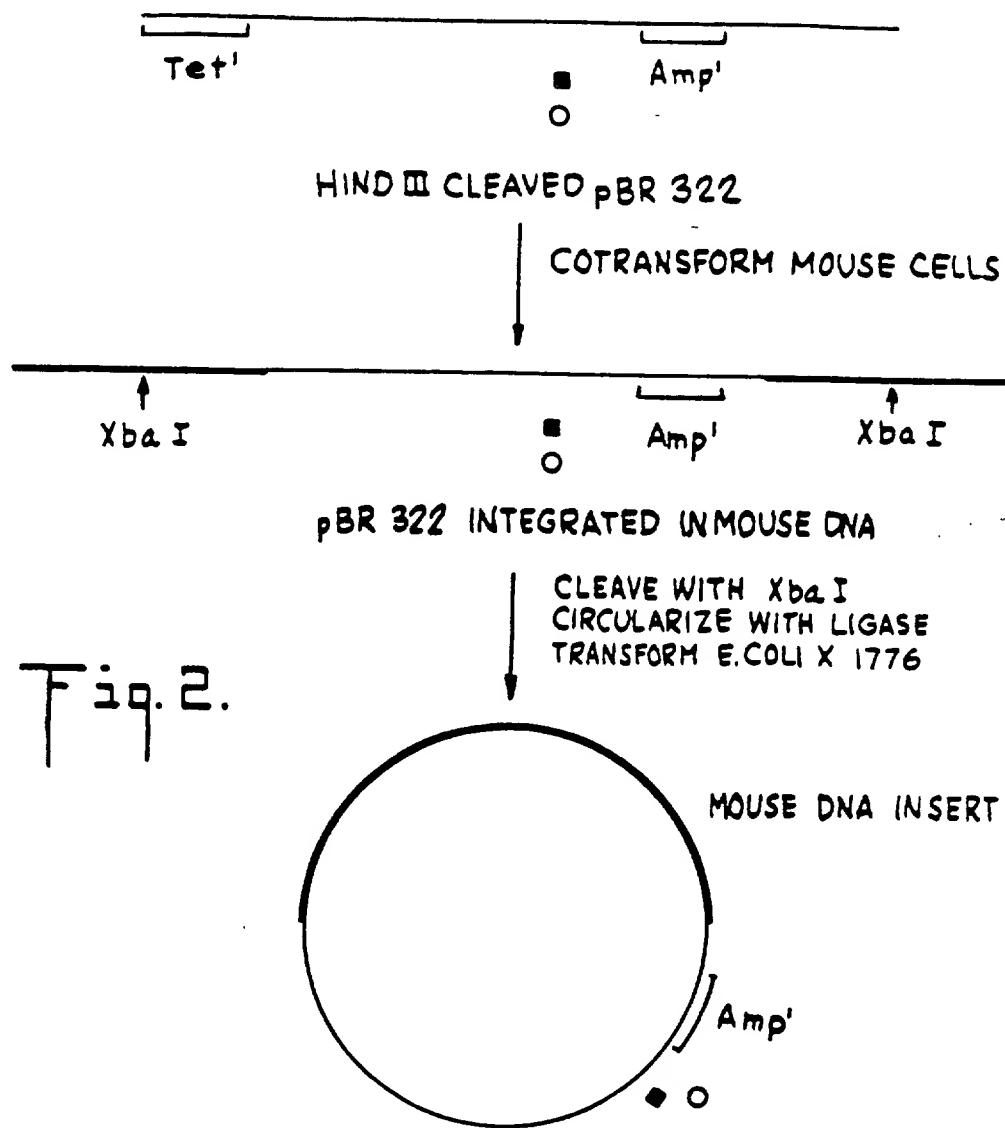
SCHEME FOR THE RESCUE OF BACTERIAL
PLASMIDS FROM TRANSFORMED CULTURED
CELLS USING DOUBLE SELECTION TECHNIQUES.

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PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

FIELD OF THE INVENTION

This invention concerns the introduction and expression of genetic informational material, i.e., DNA which includes genes coding for proteinaceous materials and-/or genes regulating or otherwise influencing the production thereof, into eucaryotic cells, that is, cells of organisms classified under the Superkingdom Eucaryotes including organisms of the Plant and Animal Kingdoms. Such genetic intervention is commonly referred to as genetic engineering and in certain aspects involves the use of recombinant DNA technology. The invention disclosed is to be distinguished from the introduction of DNA into organisms of the Superkingdom Procaryotes including particularly bacteria. This distinction is based in part upon the basic differences between eucaryotic and procaryotic cells, the former being characterized by true nuclei formed by nuclear envelopes and by meiosis and the latter being characterized by the absence of well-defined nuclei and the absence of meiosis. Moreover, at the genetic level many genes in eucaryotes are split by non-coding sequences which are not continuously colinear, whereas in procaryotes, the genes are continuously colinear.

BACKGROUND OF THE INVENTION

Although advances in the understanding of procaryotic organisms, particularly bacteria, having for the most part proceeded independently of advances in the understanding of eucaryotic organisms, it may be helpful to an appreciation of the present invention to set forth certain developments involving procaryotes.

In 1944, Avery reported the transformation of a procaryotic cell using DNA-mediated transfer of a cellular gene. Avery, O. T., et al., J. Exp. Med. 79: 137-158 (1944). Thereafter, reports of procaryotic transformation occurred in the literature. In 1975, Cohen and others reported results involving first transformation, then cotransformation of the procaryote Escherichia coli. Kretschmer, P. J., et al., J. Bacteriology 124: 225-231 (1975). In the experiments reported therein the authors disclosed the cotransformation of procaryotic cells using plasmid DNA, that is, extrachromosomal DNA which occurs naturally in many strains of Enterobacteriacae. In these experiments it was found that particular cells in a CaCl₂-treated bacterial population are preferentially competent for tranformation. However, the frequency of transformation and the stability of the transformants obtained was low, possibly because the plasmid is not incorporated into the chromosomal DNA. As a result, cotranformants lost acquired traits after several generations. In addition, these experiments with bacteria required the addition of a gene promoter to the transforming DNA in order to obtain expression.

Meanwhile, experiments with eucaryotic cells proceeded substantially independently of those with procaryotic cells. In 1962, Szybalska, E. H. and Szybalski, W. PNAS 48: 2026 (1962) reported the transformation of mammlian cells but with such low frequency of trans-

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formation that it was not possible to distinguish tranformants from cells which had merely undergone spontaneous reversion. Again, as with procaryotic cells, further reports of eucaryotic transformation occurred in the literature, but such results were oftentimes not reproducible by others. In addition, low frequencies of transformation, lack of understanding of the molecular basis for gene expression and the lack of molecular hybridization probes contributed to the lack of progress in this area. As a result, studies on the transformation of eucaryotic cells were essentially restricted to viral genes. Graham, F. L., et al., Cold Spring Harbor Symp. Quant. Biol. 39: 637-650 (1975) and McCutchen, J. H. and Pagano, J. S., Journal National Cancer Institute, 41: 351-357 (1968).

More recently, however, eucaryotic cells, specifically mammalian cells, were transformed with foreign DNA coding for a selectable phenotype. Wigler, M., et al., Cell 11: 223-232 (1977). This work has been extended and has resulted in the present invention wherein it has been discovered inter alia that eucaryotic cells can be cotransformed to yield transformants having foreign DNA integrated into the chromosomal DNA of the eucaryotic cell nucleus. Moreover, it has unexpectedly been discovered that such foreign DNA can be expressed by the cotransformants to generate functional proteins. In addition, by contrast with procaryotic transformants, the foreign DNA is stably expressed through hundreds of generations, a result that may be attributable to integration of the foreign DNA into the chromosomal DNA.

The present invention provides major advances over bacterial systems for future use in the commercial preparation of proteinaceous materials particlarly proteins of eucaryotic origin such as interferon protein, antibodies, insulin, and the like. Such advantages include the ability to use unaltered genes coding for precursors for such proteinaceous materials. After cellular synthesis, the precursor can be further processed or converted within the eucaryotic cell to produce the desired molecules of biological significance. This phenomenon is well known for insulin which is initially produced in the eucaryotic cell as preproinsulin which is then converted to active insulin within the cell by appropriate peptide cleavage. Since procaryotic cells lack the requisite cellular machinery for converting preproinsulin to insulin, the insertion into a procaryotic cell of the eucaryotic gene associated with insulin will result in the production of preproinsulin, not insulin. Although, in the case of insulin, a relatively small and well characterized protein, this difficulty can be overcome by chemical synthesis of the appropriate gene, such an approach is inherently limited by the level of understanding of the amino acid sequence of the desired protein. Thus, for interferon protein, clotting factors, antibodies and uncharacterized enzymes, for which the exact amino acid sequence is not yet known, a procaryotic system will likely not prove satisfactory. By contrast, a eucaryotic system is not associated with such disadvantages since the eucaryotic cell possesses the necessary processing machinery. It is thus one important object of the present invention to provide a process for producing desired proteinaceous materials such as interferon protein, insulin, antibodies and the like which does not require a detailed molecular understanding of amino acid sequence.

In addition to the problem of precursors having additional amino acids which must be removed to produce

active protein, important biological materials may be modified by chemical additions after synthesis and cleavage. Thus, for example, human-produced interferon is a glycoprotein containing sugar molecules in addition to protein. If produced in a bacterial cell, the interferon lacks the sugar molecules which are added when interferon is produced in a human cell. Moreover, proteinaceous materials produced within bacteria may include endotoxins which can cause inflammation if the proteinaceous material is administered to a mammal without significant purification. By contrast, interferon produced in a eucaryotic cell would be free of endotoxins.

It is therefore another important object of this invention to provide a process for producing compounds which include both non-proteinaceous and proteinaceous moieties such as glycoproteins which cannot be produced in bacterial cell.

SUMMARY OF THE INVENTION

This invention provides a process for inserting foreign DNA into eucaryotic cells by cotransforming the cells with this foreign DNA and with unlinked DNA which codes for proteinaceous material associated with a selectable phenotype not otherwise expressed by the cell. The cotransformation is carried out in a suitable medium and in the presence of selective conditions permitting survival and/or identification of eucaryotic cells which have acquired the selectable phenotype. The process of this invention is particularly suited for the insertion into eucaryotic cells of DNA which codes for proteinaceous materials which are not associated with a selectable phenotype such as interferon protein, insulin, growth hormones clotting factors, viral antigens, antibodies and certain enzymes.

By use of the cotransformation process of the present invention is it possible to produce eucaryotic cells which synthesize desired proteinaceous and other materials and which can be grown in culture to produce these materials in quantities not obtainable with conventional technology.

In one embodiment of the invention, the cotransformation process can be used to insert multiple copies of genes coding for desired materials into eucaryotic cells. Alternatively, a multiplicity of foreign DNA molecules corresponding to multiple copies of a desired gene can be inserted into eucaryotic cells by transformation with molecules each of which is formed by linking a foreign DNA molecule to a second DNA molecule corresponding to an amplifiable gene for a dominant selectable phenotype not otherwise expressed by the cell. The transformation is then carried out in the presence of successively elevated concentrations of an agent permitting survival and/or identification of eucaryotic cells which have acquired multiple copies of the amplifiable gene. This approach is particularly useful when the dominant selectable phenotype is resistance to a drug which is lethal unless multiple copies of the drug resistant gene are present and the agent is the drug.

By inserting multiple copies of genes coding for desired materials into eucaryotic cells according to either of these approaches it is possible to produce eucaryotic cells which yield desired materials in high concentrations and which can be grown in culture to produce such materials in quantities not obtainable with conventional technology.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic flow diagram illustrating the cotransformation process in accordance with the present invention.

FIG. 2 is a schematic flow diagram illustrating a process for recovering foreign DNA I from cotransformed cultured cells using double selection techniques.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Transformation means the process for changing the genotype of a recipient cell mediated by the introduction of purified DNA. Transformation is typically detected by a stable and heritable change in the phenotype of the recipient cell that results from an alteration in either the biochemical or morphological properties of the recipient cell.

Cotransformation means the process for carrying out transformations of a recipient cell with more than one different gene. Cotransformation includes both simultaneous and sequential changes in the genotype of a recipient cell mediated by the introduction of DNA corresponding to either unlinked or linked genes.

Proteinaceous material means any biopolymer formed from amino acids.

Genotype means the genetic constitution of an organism as distinguished from its physical appearance.

Phenotype means the observable properties of an organism as produced by the genotype in conjunction with the environment.

Selectable phenotype is a phenotype which confers upon an organism ability to exist under conditions which kill off all organisms not possessing the phenotype. Examples include drug resistance or the ability to synthesize some molecule necessary to cell metabolism in a given growth medium. As used herein, selectable phenotypes also include identifiable phenotypes such as the production of materials which pass from or are secreted by the cell and can be detected as new phenotypes either by functional, immunologic or biochemical assays.

Interferon protein means the proteinaceous part of the glycoprotein interferon, that is, the portion remaining after removal of the sugar portion. It includes the protein portion of interferon derived from human leukocyte, fibroblast or lymphoblastoid cells.

Chromosomal DNA means the DNA normally associated with histone in the form of chromosomes residing in the nucleus of a eucaryotic cell.

Transcription means the formation of a RNA chain in accordance with the genetic information contained in the DNA.

Translation means the process whereby the genetic information in an mRNA molecule directs the order of specific amino acids during protein synthesis.

In accordance with the present invention, foreign DNA I can be inserted into any eucaryotic cell by cotransforming the cell with DNA I and with unlinked foreign DNA II which includes a gene coding for a selectable phenotype not expressed by the cell unless acquired by transformation. The cotransformation is carried out in a suitable growth medium and in the presence of selective conditions such that the only cells

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which survive or are otherwise altered are those which have required the selectable phenotype. See FIG. 1.

Although the experiments discussed hereinafter concern cultured eucaryotic cells of mammalian origin such as human blood cells, mouse fibroblast cells, chinese hamster ovary cells and mouse teratocarcinoma cells, it is clear that the process described is generally applicable to all eucaryotic cells including, for example, cells from birds such as chickens, cells from yeast and fungi, and cells from plants including grains and flowers. Therefore, it is to be understood that the invention encompasses all eucaryotic cells even though the invention may ultimately be most useful in cotransforming mammalian cells.

The present invention is especially useful in connection with the insertion into eucaryotic cells of foreign DNA which includes genes which code for proteinaceous materials not associated with selectable phenotypes. Since such proteinaceous materials are characterized by the fact that they are not associated with a selectable phenotype, cells which contain DNA coding therefore cannot be identified except by destruction of the transformed cell and examination of its contents.

Examples of proteinaceus materials, the genes for which may be inserted into and expressed by eucaryotic cells using the cotransformation process include interferon protein, insulin, growth hormones, clotting factors, viral antigens, enzymes and antibodies.

Although in some cases the DNA I and DNA II may not need to be purified to obtain integration and expression, it is oftentimes preferable that the DNAs be purified prior to use in cotransforming cells. Such purification limits the possibility of spurious results due to the presence of contaminants and increases the probability that cotransformed cells can be identified and stably cultured. Also, although not essential, it is sometimes desirable that DNA I and/or DNA II have been obtained by restriction endonuclease cleavage of chromosomal donor DNAs, such as, for example, restriction endonuclease cleavage of eucaryotic chromosomal DNA. Additionally, it is preferable that DNA I and DNA II be treated with calcium phosphate prior to use in cotransforming eucaryotic cells. The procedure for so treating DNA with calcium phosphate is set forth more fully hereinafter. Finally, it is preferable that the foreign DNA I be present during cotransformation in an amount relative to DNA II coding for a selectable phenotype which constitutes an excess of the former, such as an amount in the range from about 1:1 to about 100,000:1.

In a preferred embodiment of the invention, the foreign DNA I and/or the foreign DNA II are attached to bacterial plasmid or phage DNA prior to use in cotransforming eucaryotic cells. In a particularly promising embodiment, foreign DNA I and/or DNA II are attached to phage DNA and then encapsidated in phage particles prior to cotransformation.

Although any DNA II coding for a selectable phenotype would be useful in the cotransformation process of the present invention, the experimental details set forth particularly concern the use of a gene for thymidine kinase obtained from herpes simplex virus and the use of a gene for adenine phosphoribosyl transferase. In addition, a DNA II which includes a gene coding for a selectable phenotype associated with drug resistance, e.g., a mutant dihydrofolate reductase gene which renders cells resistant to methotrexate greatly extends the applicability of the process.

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In accordance with a preferred embodiment, the cotransformation involves DNA I which is physically and chemically unlinked to DNA II, and the DNA I is stably integrated into the chromosomal DNA within the nucleus of the cotransformed eucaryotic cell.

Cotransformation in accordance with this invention may be carried out in any suitable medium limited only in that cotransformed cells be capable of survival and/or identification on the medium. Merely by way of example, a suitable medium for mouse fibroblast cells which have aquired the thymidine kinase gene is HAT described more fully hereinafter Also, the cotransformation is carried out in the presence of selective conditions which permit survival and/or identification of those cells which have acquired the selectable phenotype. Such conditions may include the presence of nutrients, drug or other chemical antagonists, temperature and the like.

Eucaryotic cells cotransformed in accordance with this invention contain foreign DNA I coding for desired materials which can be recovered from the cells using techniques well known in the art. Additionally, the cells can be permitted to transcribe DNA I to form mRNA which in turn is translated to form protein or other desired material which may be recovered. again using well known techniques. Finally, the cells can be grown in culture, harvested and protein or other desired material recovered therefrom.

Although the desired proteinaceous materials identified hereinabove are natural materials, the process can be equally useful in the production of synthetic biopolymers for which synthetic genes are constructed. Thus, the instant invention provides a process for producing novel proteins not yet in existence. Additionally, it provides a process for producing proteins which, although they presently exist, do so in such minute quantities or in such impure form that their isolation and/or identification cannot otherwise be effected. Finally, the invention provides a process for producing partially proteinaceous products such as the glycoproteins and other products, the synthesis of which is genetically directed.

Another aspect of the invention involves processes for inserting multiple copies of genes into eucaryotic cells in order to increase the amount of gene product formed within the cell. One process for inserting a multiplicity of foreign DNA I molecules into a eucaryotic cell comprises cotransforming the cell with multiple DNA I molecules and with multiple, unlinked foreign DNA II molecules corresponding to multiple copies of an amplifiable gene for a dominant selectable phenotype not otherwise expressed by the cell. This cotransformation process is carried out in a suitable medium and in the presence of an agent permitting survival and/or identification of cells which acquire the dominant selectable phenotype. Preferably, this is done in the presence of successively higher concentrations of such an agent so that only those cells acquiring the highest number of amplifiable dominant genes (DNA II) survive and/or are identified. These cells then also contain multiple copies of DNA I. This approach is particularly appropriate for the insertion of multiple copies of amplifiable genes which confer drug resistance upon the cell, e.g., the mutant dihydrofolate reductase gene which renders cells resistant to methotrexate.

Cotransformed eucaryotic cells which have acquired multiple copies of DNA I may then be used to produce

increased amounts of the gene product for which DNA I codes in the same manner as described hereinabove.

Alternatively, multiple copies of foreign genes can be generated in and ultimately expressed by eucaryotic cells by transforming the eucaryotic cells with DNA molecules, each of which has been formed by linking a foreign DNA I to a foreign DNA II which corresponds to an amplifiable gene for a dominant selectable phenotype not normally expressed by the eucaryotic cell. The linkage between DNA I and DNA II is preferably in the form of a chemical bond, particularly a bond formed as a result of enzymatic treatment with a ligase. Transformation with such hybrid DNA molecules so formed is then carried out in a suitable growth medium and in the presence of successively elevated concentrations, e g., amounts ranging from 1:1 to 10,000:1 on a molarity basis, of an agent which permits survival and/or identification of those eucaryotic cells which have acquired a sufficiently high number of copies of the amplifiable gene. Using this approach, eucaryotic cells which have acquired multiple copies of the amplifiable gene for a dominant selectable phenotype not otherwise expressed by the cell survive and/or are identifiable in the presence of elevated concentrations of an agent complementary to the amplifiable gene which would otherwise result in death or inability to identify the cells.

Although various amplifiable genes for dominant selectable phenotypes are useful in the practices of this invention, genes associated with drug resistance, e.g., the gene for dihydrofolate reductase which renders cells resistant to methotrexate, are particularly suitable.

By using either of the two approaches just described, multiple copies of proteinaceous or other desired molecules can be produced within eucaryotic cells. Thus, for example, multiple molecules of interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody or of interferon per se can be produced by eucaryotic cells, particularly mammalian cells, which have been transformed using hybrid DNA or cotransformed using purified DNA which has been treated with calcium phosphate in the manner described hereinafter. Thus, this invention provides a process for producing highly desired, rare and costly proteinaceous and other biological materials in concentrations not obtainable using conventional techniques.

Still another aspect of the present invention involves the preparation of materials normally produced within eucaryotic cells in minute amounts such as glycoprooteins including interferon, which are in part protein but additionally include other chemical species such as sugars, ribonucleic acids, histones and the like. Although the method or methods by which cells synthesize complicated cellular materials such as the glycoproteins are poorly understood, it is anticipated that by using the process of the present invention it will be possible to synthesize such materials in commercially useful quantities. Specifically, it is anticipated that after inserting a gene or genes for the protein portion of a cellular material such as a glycoprotein, which includes a non-protein portion, into a eucaryotic cell of the type which normally produces such material, the cell will not only produce the corresponding proteinaceous material but will utilize already existing cellular mechanisms to process the proteinaceous materials, if and to the extent necessary, and will also add the appropriate non-proteinaceous material to form the complete, biologically active material. Thus, for example, the complete biologically active glyprotein, interferon, could be

prepared by synthesizing interferon protein in the manner described and additionally permitting the cell to produce the non-proteinaceous or sugar portion of interferon and to synthesize or assemble true interferon therefrom. The interferon so prepared could then be recovered using conventional techniques.

In accordance with the present invention and as described more fully hereinafter, eucaryotic cells have been stably transformed with precisely defined procaryotic and eucaryotic genes for which no selective criteria exist. The addition of a purified viral thymidine kinase (tk) gene to mouse cells lacking this enzyme results in the appearance of stable transformants which can be selected by their ability to grow in HAT medium. Since these biochemical transformants might represent a subpopulation of competent cells which are likely to integrate other unlinked genes at frequencies higher than the general population; contransformation experiments were performed with the viral tk gene and bacteriophage ΦX174, plasmid pBR 322 or cloned chromosomal human or rabbit β-globin gene sequences. Tk transformants were cloned and analyzed for contransfer of additional DNA sequences by blot hybridization. In this manner, mouse cell lines were identified which contain multiple copies of ΦX, pBR 322, or human and rabbit β-globin sequences. From one to more than 50 cotransformed sequences are integrated into high molecular weight DNA isolated from independent clones. Analysis of subclones demonstrates that the contransformed DNA is stable through many generations in culture. This cotransformation system allows the introduction and stable integration of virtually any defined gene into cultured eucaryotic cells. Ligation to either viral vectors or selectable biochemical markers is not required.

Cotransformation with dominant-acting markers should in principle permit the introduction of virtually any cloned genetic element into wild-type cultured eucaryotic cells. To this end, a dominant-acting, methotrexate resistant, dihydrofolate reducatse gene from CHO A29 cells was transferred to wild-type cultured mouse cells. By demonstrating the presence of CHO DHFR sequences in transformants, definitive evidence for gene transfer was provided. Exposure of these cells to elevated levels of methotrexate results in enhanced resistance to this drug, accompanied by amplification of the newly transferred gene. The mutant DHFR gene, therefore, has been used as a eucaryotic vector, by ligating CHO A29 cell DNA to pBR 322 sequences prior to transformation. Amplification of the DHFR sequences results in amplification of the pBR sequences. The use of this gene as a dominant-acting vector in eucaryotic cells will expand the repetoire of potentially transformable cells, no longer restricting these sort of studies to available mutants.

Using the techniques described, the cloned chromosomal rabbit β-globin gene has been introduced into mouse fibroblasts by DNA-mediated gene transfer. The cotransformed mouse fibroblast containing this gene provides a unique opportunity to study the expression and subsequent processing of these sequences in a heterologous host. Solution hybridization experiments in concert with RNA blotting techniques indicate that in at least one transformed cell line rabbit globin sequences are expressed in the cytoplasm as a polyadenylated 9S species. These 9S sequences result from perfect splicing and removal of the two intervening sequences. These results therefor suggest that nonerythroid cells from

heterologous species contain the enzymes necessary to correctly process the intervening sequences of a rabbit gene whose expression is usually restricted to erythroid cells. Surprisingly, however, 45 nucleotides present at the 5' terminus of mature rabbit mRNA are absent from the globin mRNA sequence detected in the cytoplasm of the transformants examine. These studies indicate the potential value of cotransformation systems in the analysis of eucaryotic gene expression. The introduction of wild type genes along with native and in vitro constructed mutant genes into cultured cells provides an assay for the functional significance of sequence organization.

Recombinant DNA technology has facilitated the isolation of several higher eucaryotic genes for which hybridization probes are available. Genes expressed at exceedingly low levels, with mRNA transcripts present at from one to 20 copies per cell, such as those genes coding for essential metabolic functions, cannot be simply isolated by conventional techniques involving construction of cDNA clones and the ultimate screening of recombinant libraries. An alternative approach for the isolation of such rarely expressed genes has therefore been developed utilizing transformation in concert with a procedure known as plasmid rescue. This schema which is currently underway in the laboratory is outlined below. The aprt gene of the chicken is not cleaved by the enzyme, Hin III or Xba, and transformation of aprt⁻mouse cells with cellular DNA digested with these enzymes results in the generation of aprt⁺clonies which express the chicken aprt genes. Ligation of Hin III-cleaved chicken DNA with Hin III-cleaved plasmid pBR 322 results in the formation of hybrid DNA molecules in which the aprt gene is now adjacent to plasmid sequences. Transformation of aprt⁻cells is now performed with this DNA. Transformants should contain the aprt gene convalently linked to pBR 322 with this entire complex integrated into high molecular weight DNA in the mouse cell. This initial cellular transformation serves to remove the chicken aprt gene from the vast majority of other chick sequences. This transformed cell DNA is now treated with an enzyme, Xba I, which does not cleave either pBR 322 or the aprt gene . The resultant fragments are then circularized with ligase. One such fragment should contain the aprt gene covalently linked to pBR 322 sequences coding for an origin of replication and the ampicillin resistant marker. Transformation of a bacterium such as E. coli with these circular markers selects for plasmid sequences from eucaryotic DNA which are now linked to chicken aprt sequences. This double selection technique should permit the isolation of genes expressed at low levels in eucaryotic cells for which hybridization probes are not readily obtained.

In order to assist in a better understanding of the present invention, the results of various experiments are now set forth.

EXPERIMENTAL DETAILS
FIRST SERIES OF EXPERIMENTS

The identificaton and isolation of cells transformed with genes which do not code for selectable markers is problematic since current transformation procedures are highly inefficient. Thus, experiments were undertaken to determine the feasibility of cotransforming cells with two physically unlinked genes. In these experiments it was determined that cotransformed cells could be identified and isolated when one of the genes

codes for a selectable marker. Viral thymidine kinase gene was used as a selectable marker to isolate mouse cell lines which contain the tk gene along with either bacteriophage ΦX 174, plasmid pBR 322 or cloned rabbit β-globin gene sequences stably integrated into cellular DNA. The results of these experiments are also set forth in Wigler, M., et al., Cell 16; 777-785 (1979) and Wold, B. et al., Proc. Nat'l. Acad. Sci. 76; 5684-5688 (1979) are as follows:

EXPERIMENTAL DESIGN

The addition of the purified thymidine kinase (tk) gene from herpes simplex virus to mutant mouse cells lacking tk results in the appearance of stable transformants expressing the viral gene which can be selected by their ability to grow in HAT. Maitland, N. J. and McDougall J. K. Cell, 11: 233-241 (1977); Wigler, M. et al., Cell 11: 223-232 (1977). The obtain cotransformants, cultures are exposed to the tk gene in the presence of an excess of a well-defined DNA sequence for which hybridization probes are available. Tk transformants are isolated and scored for the cotransfer of additional DNA sequences by molecular hybridization.

Cotransformation of Mouse Cells with ΦX174 DNA

ΦX174 DNA was initially used in cotransformation experiments with the tk gene as the selectable marker. ΦX replicative form DNA was cleaved with Pst 1, which recognizes a single site in the circular genome. Sanger, F. et al., Nature 265: 687-695 (1977). 500 pg of the purified tk gene were mixed with 1-10 μg of Pst-cleaved ΦX replicative form DNA. This DNA was then added to mouse Ltk⁻cells using the transformation conditions described under Methods and Materials hereinafter. After 2 weeks in selective medium (HAT), tk⁺transformants were observed at a frequency of one colony per 10 cells per 20 pg of purified gene. Clones were picked and grown to mass culture.

It was then asked whether tk⁺transformants also contained ΦX DNA sequences. High molecular weight DNA from the transformants was cleaved with the restriction endonuclease Eco RI, which recognizes no sites in the ΦX genome. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nick-translated ³²P-ΦX DNA (blot hybridization). Southern, R. M., J. Mol. Biol. 98: 503-517 (1975); Botchan, M., et al., Cell 9: 269-287 (1976); Pellicer, A., et al. Cell 14: 133-141 (1978). These annealing experiments demonstrate that six of the seven transformants had acquired bacteriophage sequences. Since the ΦX genome is not cut by the enzyme Eco RI, the number of bands observed reflects the minimum number of eucaryotic DNA fragments containing information homologous to ΦX. The clones contain variable amounts of ΦX sequences. Clones ΦX1 and ΦX2 reveal a single annealing fragment which is smaller than the ΦX genome. In these clones, therefore, only a portion of the transforming sequences persist. There was also observed a tk⁺transformant (clone 101 X3) with no detectable ΦX sequences. Clones ΦX4, 5, 6, and 7 reveal numerous high molecular weight bands which are too closely spaced to count, indicating that these clones contain multiple ΦX-specific fragments. These experiments demonstrate cotransformation of cultured mammalian cells with the viral tk gene and ΦX DNA.

Selection is Necessary to identify ΦX Transformants

It was next asked whether transformants with ΦX DNA was restricted to the population of tk+cells or whether a significant proportion of the original culture now contained ΦX sequences. Cultures were exposed to a mixtures of the tk gene and ΦX DNA in a molar ratio of 1:2000 or 1:20,000. Half of the cultures were plated under selective conditions, while the other half were plated in neutral media at low density to facilitate cloning. Both selected (tk+) and unselected (tk−) colonies were picked, grown into mass culture and scored for the presence of ΦX sequences. In this series of experiments, eight of the nine tk+selected colonies contained phage information. As in the previous experiments, the clones contained varying amounts of ΦX DNA. In contrast, none of fifteen clones picked at random from neutral medium contained any ΦX information. Thus, the addition of a selectable marker facilitates the identificaton of those cells which contain ΦX DNA.

ΦX Sequences are Integrated into Cellular DNA

Cleavage of DNA from ΦX transformants with Eco RI generates a series of fragments which contain ΦX DNA sequences. These fragments may reflect multiple integration events. Alternatively, these fragments could result from tandem arrays of complete or partial ΦX sequences which are not integrated into cellular DNA. To distinguish between these possibilities, transformed cell DNA was cut with BAM HI or Eco RI, neither of which cleaves the ΦX genome. If the ΦX DNA sequences were not integrated, neither of these enzymes would cleave the ΦX fragments. If the ΦX DNA sequences were not integrated, neither of these enzymes would cleave the ΦX fragments. Identical patterns would be generated from undigested DNA and from DNA cleaved with either of these enzymes. If the sequences are integrated, then BAM HI and Eco RI should recognize different sites in the flanking cellular DNA and generate unique restriction patterns. DNA from clones ΦX4 and ΦX5 was cleaved with BAM III or Eco RI and analyzed by Southern hybridization. In each instance, the annealing pattern with Eco RI fragments differed from that observed with the BAM HI fragments. Furthermore, the profile obtained with undigested DNA reveals annealing only in very high molecular weight regions with no discrete fragments observed. Similar observations were made on clone ΦX1. Thus, the most of the ΦX sequences in these three clones are integrated into cellular DNA.

Intracellular Localization of the ΦX Sequences

The location of ΦX sequences in transformed cells was determined by subcellular fractionation. Nuclear and cytoplasmic fractions was prepared, and the ΦX DNA sequence content of each was assayed by blot hybridization. The data indicate that 95% of the ΦX sequences are located in the nucleus. High and low molecular weight nuclear DNA was prepared by Hirt fractionation. Hirt, B. J., Mol. Biol. 26: 365-369 (1967). Hybridization with DNA from these two fractions indicates that more than 95% of the ΦX informaton co-purifies with the high molecular weight DNA fraction. The small amount of hybridization observed in the supernatant fraction reveals a profile identical to that of the high molecular weight DNA, suggesting contamination of this fraction with high molecular weight DNA.

Extent of Sequence Representation of the ΦX Genome

The annealing profiles of DNA from transformed clones digested with enzymes that do not cleave the ΦX genome provide evidence that integration of ΦX sequences has occured and allow us to estimate the number of ΦX sequences integrated. Annealing profiles of DNA from transformed clones digested with enzymes which cleave within the ΦX genome allow us to determine what proportion of the genome is present and how these sequences are arranged following integration. Cleavage of ΦX with the enzyme Hpa I generates three fragments for each integration event: two "internal" fragments of 3.7 and 1.3 kb which together comprise 90% of the ΦX genome, and one "bridge" fragment of 0.5 kb which spans the Pst I cleavage site. In the annealing profile observed when clone ΦX4 is digested with Hpa I, two intense bands are observed at 3.7 and 1.3 kb. A less intense series of bands of higher molecular weight is also observed, some of which probably represent ΦX sequences adjacent to cellular DNA. These results indicate that at least 90% of the ΦX genome is present in these cells. It is worth noting that the internal 1.3 kb Hpa I fragment is bounded by an Hpa I site only 30 bp from the Pst I cleavage site. Comparison of the intensities of the internal bands with known quantities of Hpa I-cleaved ΦX DNA suggests that this clone contains approximately 100 copies of the ΦX genome. The annealing pattern of clone 5 DNA cleaved with Hpa I is more complex. If internal fragments are present, they are markedly reduced in intensity; instead, multiple bands of varying molecular weight are observed. The 0.5 kb Hpa I fragment which bridges the Pst I cleavage site is not observed for either clone ΦX4 or clone ΦX5.

A similar analysis of clone ΦX4 and ΦX5 was performed with the enzyme Hpa II. This enzyme cleaves the ΦX genome five times, thus generating four "internal" fragments of 1.7, 0.5, 0.5 and 0.2 kb, and a 2.6 kb "bridge" fragment which spans the Pst I cleavage site. The annealing patterns for Hpa II-cleaved DNA from ΦX clones 4 and 5 each show an intense 1.7 kb band, consistent with the retention of at least two internal Hpa II sites. The 0.5 kb internal fragments can also be observed, but they are not shown on this gel. Many additional fragments, mostly of high molecular weight, are also present in each clone. These presumably reflect the multiple integration sites of ΦX DNA in the cellular genome. The 2.6 kb fragment bridging the Pst I cleavage site, however, is absent from clone ΦX4. Reduced amounts of annealing fragments which co-migrate with the 2.6 kb Hpa II bridge fragment are observed in clone ΦX5. Similar observations were made in experiments with the enzyme Hae III. The annealing pattern of Hae III-digested DNA from these clones was determined. In accord with previous data, the 0.87 kb Hae III bridge fragment spanning the Pst site is absent or present in reduced amount in transformed cell DNA. Thus, in general, "internal" fragments of ΦX are found in these transforments, while "bridge" fragments which span the Pst I cleavage site are reduced or absent.

Stability of the Transformed Genotype

Previous observations on the transfer of selectable biochemical markers indicate that the transformed phenotype remains stable for hundreds of generations if cells are maintained under selective pressure. If maintained in neutral medium, the transformed phenotype is

lost at frequencies which range from 0.1 to as high as 30% per generation. Wigler, M., et al., Cell 11; 223-232 (1977); Wigler, M. et al., PNAS 76: 5684-.088 (1979). The use of transformation to study the expression of foreign genes depends upon the stability of the transformed genotype. This is an important consideration with genes for which no selective criteria are available. It was assumed that the presence of ΦX DNA in transformants confers no selective advantage on the recipient cell. Therefore, the stability of the ΦX genotype was examined in the descendants of two clones after numerous generations in culture. Clone ΦX4 and ΦX5, both containing multiple-copies of ΦX DNA, were subcloned and six independent subclones from each clone were picked and grown into mass culture. DNA from each of these subclones from each original clone were picked and grown into mass culture. DNA from each of these subclones was then digested with either Eco RI or Hpa I, and the annealing profiles of ΦX-containing fragments were compared with those of the original parental clone. The annealing pattern observed for four of the six ΦX4 subclones is virtually identical to that of the parent. In two subclones, an additional Eco RI fragment appeared which is of identical molecular weight in both. This may have resulted from genotypic heterogeneity in the parental clone prior to subcloning. The patterns obtained for the subclones of ΦX5 are again virtually identical to the parental annealing profile. These data indicate that ΦX DNA is maintained within the ten subclones examined for numerous generations without significant loss or translocation or information.

Integration of pBR322 into Mouse Cells

The observations in cotransformation have been extended to the EK2-approved bacterial vector, plasmid pBR322. pBR322 linearized with BAM HI was mixed with the purified viral tk gene in a molar ratio of 1000:1. Tk+transformants were selected and scored for the presence of pBR322 sequences. Cleavage of BAM HI linearized pBR322 DNA with Bgl I generates two internal fragments of 2.4 and 0.3 kb. The sequence content of the pBR322 transformants was determined by digestion of transformed cell DNA with Bgl I followed by annealing with ^{32}p-labeled plasmid DNA. Four of five clones screened contained the 2.4 kb internal fragment. The 0.3 kb fragment would not be detected on these gels. From the intensity of the 2.4 kb band in comparison with controls, we conclude that multiple copies of this fragment are present in these transformants. Other bands are observed which presumably represent the segments of pBR322 attached to cellular DNA.

Transformation of Mouse Cells with the Rabbit β-Globin Gene

Transformation with purified eucaryotic genes may provide a means for studying the expression of cloned genes in a heterologous host. Cotransformation experiments were therefore performed with the rabbit β major globin gene which was isolated from a cloned library of rabbit chromosomal DNA (Maniatis, T, et al., Cell 15: 687-701 (1978). One β-globin clone designated RβG-1 consists of a 15 kb rabbit DNA fragment carried on the bacteriophage cloning vector Charon 4a. Intact DNA from this clone (RβG-1) was mixed with the viral tk DNA at a molar ratio of 100:1, and tk+ transformants were isolated and examined for the presence of rabbit globin sequences. Cleavage of RβG-1

with the enzyme Kpn I generates a 4.7 kb fragment which contains the entire rabbit β-globin gene. This fragment was purified by gel electrophoresis and nick-translated to generate a probe for subsequent annealing experiments. The β-globin genes of mouse and rabbit are partially homologous, although we do not observe annealing of the rabbit β-globin probe with Kpn-cleaved mouse DNA under our experimental conditions. In contrast, cleavage of rabbit liver DNA with Kpn I generates the expected 4.7 kb globin band. Cleavage of transformed cell DNA with the enzyme Kpn I generates a 4.7 kb fragment containing globin-specific information in six of the eight tk+ transformants examined. In two of the clones, additional rabbit globin bands are observed which probably result from the loss of at least one of the Kpn sites during transformation. The number of rabbit globin genes integrated in these transformants is variable. In comparison with controls, some clones contain a single copy of the gene, while others contain multiple copies of this heterologous gene. These results demonstrate that cloned eucaryotic genes can be introduced into cultured mammalian cells by cotransformation.

TRANSFORMATION COMPETENCE IS NOT STABLY INHERITED

Our data suggest the existenc of a subpopulation of transformation-competent cells within the total cell population. If competence is a stably inherited trait, then cells selected for transformation should be better recipients in subsequent gene transfer experiments than their parental cells. Two results indicate that as in procaryotes, competence is not stably heritable. In the first series of experiments, a double mutant, Ltk aprt⁻ (deficient in both tk and aprt), was transformed to either the tk+ aprt⁻ or the tk⁻ aprt+ phenotype using cellular DNA as donor. Wigler, M. et al., Cell 14: 725-731 (1978); Wigler, M. et al., PNAS 76: 5684-5688 (1979) These clones were then transformed to the tk+ aprt+ phenotype. The frequency of the second tmasformation was not significantly higher than the first. In another series of experiments, clones ΦX4 and ΦX5 were used as recipients for the transfer of a mutant folate reductase gene which renders recipient cells resistant to methotrexate (mtx). The cell line A29 Mtx^RIII contains a mutation in the structural gene for dihydrofolate reductase, reducing the affinity of this enzyme for methotrexate. Flintoff, W. F. et al., Somatic Cell Genetic 2: 245-261 (1976). Genomic DNA from this line was used to transform clones ΦX4 and ΦX5 and Ltk⁻ cells. The frequency of transformation to mtx resistance for the ΦX clones was identical to that observed with the parental Ltk⁻ cells. It is therefore concluded that competence is not a stably heritable trait and may be a transient property of cells.

DISCUSSION

In these studies, we have stably transformed mammalian cells with precisely defined procaryotic and eucaryotic genes for which no selective criteria exist Our chosen design derives from studies of tramsformation in bacteria which indicate that a small but selectable subpopulation of cells is competent in transformation Thomas, R. Biochim. Biophys. Acta 18: 467-481 (1955). Hotchkiss, R. PNAS 40: 49-55 (1959); Thomasz, A and Hotchkiss R. PNAS 51: 480-487 (1964); Spizizen. J et al., Ann Rev. Microbiol. 20: 371-400 (1966) If this is also true for animal cells, then biochemical transfor-

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mants will represent a subpopulation of competent cells which are likely to integrate other unlinked genes at frequencies higher than the general population. Thus, to identify transformants containing genes which provide no selectable trait, cultures were cotransformed with a physically unlinked gene which provided a selectable marker. This cotransformation system should allow the introduction and stable integration of virtually any defined gene into cultured cells. Ligation to either viral vectors or selectable biochemical markers is not required.

Cotransformation experiments were performed using the HSV tk gene as the selectable biochemical marker. The addition of this purified tk gene to mouse cells lacking thymidine kinase results in the appearance of stable transformants which can be selected by their ability to grow in HAT. Tk+ transformants were cloned and analyzed by blot hybridization for cotransfer of additional DNA sequences. In this manner, we have constructed mouse cell lines which contain multiple copies of ΦX, pBR322 an ribbit β-globin gene sequences.

The suggestion that these observations could result from contaminating procaryotic cells in our cultures is highly improbable. At least one of the rabbit β-globin mouse transformants expresses polyadenylated rabbit β-globin RNA sequences as a discrete 9S cytoplasmic species. The elaborate processing events required to generate 9S globin RNA correctly are unlikely to occur in procaryotes.

The ΦX cotransformants were studied in greatest detail. The frequency of cotransformation is high: 14 of 16 tk+ transformants contain ΦX sequences. The ΦX sequences are integrated into high molecular weight nuclear DNA. The number of integration events varies from one to more than fifty in independent clones. The extent of the bacteriophage genome present within a given transformant is also variable; while some clones have lost up to half the genome, other clones contain over 90% of the ΦX sequences. Analysis of subclones demonstrates that the ΦX genotype is stable through many generations in culture. Similar conclusions are emerging from the characterization of the pBR322 and globin gene cotransformants.

Hybridization analysis of restriction endonuclease-cleaved transformed cell DNA allows one to make some preliminary statements on the nature of the integration intermediate. Only two ΦX clones have been examined in detail. In both clones, the donor DNA was Pst I-linearized ΦX DNA. Attempts were made to distinguish between the integration of a linear or circular intermediate. If either precise circularization or the formation of linear concatamers had occurred at the Pst I cleavage site, and if integration occurred at random points along this DNA, one would expect cleavage maps of transformed cell DNA to mirror the circular ΦX map. The bridge fragment, however, is not observed or is present in reduced amounts in digests of transformed cell DNA with three different restriction endonucleases. The fragments observed are in accord with a model in which ΦX DNA integrates as a linear molecule. Alternatively, it is possible that intramolecular recombination of ΦX DNA occurs, resulting in circularization with deletions at the Pst termini. Lai, C. J. and Nathans, D. Cold Spring Harbor Symp. Quant. Biol. 39: 53-60 (1974). Random integration of this circular molecule would generate a restriction map similar to that observed for clones ΦX4 and ΦX5. Other more

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complex models of events occurring before, during or after integration can also be considered. Although variable amounts of DNA may be deleted from termini during transformation, most copies of integrated ΦX sequences in clone ΦX4 retain the Hpa I site, which is only 30 bp from the Pst I cleavage site. Whatever the mode of integration, it appears that cells can be stably transformed with long stretches of donor DNA. Transformants have been observed containing continuous stretches of donor DNA 50 kb long.

There have been attempts to identify cells transformed with ΦX sequences in the absence of selective pressure. Cultures were exposed to ΦX and tk DNA and cells were cloned under nonselective conditions. ΦX sequences were absent from all fifteen clones picked. In contrast, 14 of 16 clones selected for the tk+ phenotype contained ΦX DNA. The simplest interpretation is that a subpopulation of cells within the culture is competent in the uptake and integration of DNA. In this subpopulation of cells, two physically unlinked genes can be introduced into the same cell with high frequency. At present one can only speculate on the biological basis of competence. Competent cells may be genetic variants within the culture; however, these studies indicate that the competent phenotype is not stably inherited. If one can extrapolate from studies in procaryotes, the phenomenon of competence is likely to be a complex and transient property reflecting the metabolic state of the cell.

Cotransformants contain at least one copy of the tk gene and variable amounts of ΦX DNA. Although transformation was performed with ΦX and tk sequences at a molar ratio of 1000:1, the sequence ratio observed in the transformants never exceeded 100:1. There may be an upper limit to the number of integration events that a cell can tolerate, beyond which lethal mutations occur. Alternatively, it is possible that the efficiency of transformation may depend upon the nature of the transforming fragment. The tk gene may therefor represent a more efficient transforming agent than phage DNA.

In other studies there has been demonstrated the cotransfer of plasmid pBR322 DNA into Ltk⁻ aprt⁻ cells using aprt+ cellular DNA as donor and aprt as selectable marker. Furthermore, the use of dominant acting mutant genes which can confer drug resistance will extend the host range for cotransformation to virtually any cultured cell.

The stable transfer of ΦX DNA sequences to mammalian cells serves as a model system for the introduction of defined genes for which no selective criteria exist. The tk cotransformation system has been used to transform cells with the bacterial plasmid pBR322 and the cloned rabbit β-globin gene. Experiments which indicate that several of the pBR transformants contain an uninterrupted sequence which includes the replicative origin and the gene coding for ampicillin resistance (β-lactamase), suggest that DNA from pBR transformants may transfer ampicillin resistance to E. coli. Although preliminary, these studies indicate the potential value of cotransformation in the analysis of eucaryotic gene expression.

SECOND SERIES OF EXPERIMENTS

Cotransformed mouse fibroblasts containing the rabbit β-globin gene provide an opportunity to study the expression and subsequent processing of these sequences in a heterologous host. In these experiments, we

demonstrate the expression of the transformed rabbit β-globin gene generating a discrete polyadenylated 9S species of globin RNA. This RNA results from correct processing of both intervening sequences, but lacks approximately 48 nucleotides present at the 5' terminus of mature rabbit β-globin mRNA.

Transformation of Mouse Cells with the Rabbit β-Globin Gene

We have performed cotransformation experiments with the chromosomal adult rabbit β-globin gene, using the purified herpes virus tk gene as a biochemical marker. The addition of the tk gene to mutant Ltk⁻ mouse fibroblasts results in the appearance of stable transformants that can be selected by their ability to grow in hypoxanthine/aminopterin/thymidine (HAT) medium. Cells were cotransformed with a 62-globin gene clone designated RβG1, which consists of a 15.5-kbp insert of rabbit DNA carried in the bacteriophage λcloning vector Charon 4A. The purified tk gene was mixed with a 100-fold molar excess of intact recombinant DNA from clone RβG1. This DNA was then exposed to mouse Ltk⁻ cells under transformation conditions described herein under Methods and Materials. After 2 weeks in selective medium, tk⁺ transformants were observed at a frequency of one colony per 10⁶ cells per 20 pg of tk gene. Clones were picked and grown into mass culture.

It was then asked if the tk⁺ transformants also contain rabbit β-globin sequences. High molecular weight DNA from eight tranformants was cleaved with the restriction endonuclease Knp I. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nicktranslated globin [³²P] DNA blot hybridization. Southern, E. M., J. Mol. Biol. 98: 503-517 (1975). Cleavage of this recombinant phage with the enzyme Kpn I generates a 4.7-kpb fragment that contains the entire adult β-globin gene, along with 1.4 kbp of 5' flanking information and 2.0 kbp of 3' flanking information. This fragment was purified by gel electrophoresis and nick translated to generate a hybridization probe. Blot hybridization experiments showed that the 4.7-kbp Kpn I fragment containing the globin gene was present in the DNA of six of the eight tk⁺ transformants. In three of the clones additional rabbit globin bands were observed, which probably resulted from the loss of at least one of the Kpn I sites during transformation. The number of rabbit globin genes integrated in these transformants was variable: some clones contained a single copy of the gene whereas other contained up to 20 copies of the heterologous gene. It should be noted that the β-globin genes of mouse and rabbit are partially homologous. However, we do not observe hybridization of the rabbit β-globin probe to Kpn-cleaved mouse DNA, presumably because Kpn cleavage of mouse DNA leaves the β-gene cluster in exceedingly high molecular weight fragments not readily detected in these experiments. These results demonstrate the introduction of the cloned chromosomal rabbit β-globin transfer.

RABBIT β-GLOBIN SEQUENCES ARE TRANSCRIBED IN MOUSE TRANSFORMANTS

The cotransformation sysem we have developed may provide a functional assay for cloned eucaryotic genes if these genes are expressed in the heterologous recipient cell. Six transformed cell clothes were therefor ana-

lyzed for the presence of rabbit β-globin sequences. In initial experiments, solution hybridization reactions were performed to determine the cellular concentration of rabbit globin transcripts in our transformants A radioactive cDNA copy of purified rabbit α- and β-globin mRNA was annealed with the vast excess of cellular RNA. Because homology exists between the mouse and rabbit globin sequences, it was necessary to determine experimental conditions such that the rabbit globin cDNAs did not form stable hybrids with mouse globin mRNA but did react completely with homologous rabbit sequences. At 75° C. in the presence of 0.4 M NaCl, over 80% hybridization was observed with the rabbit globin mRNA, whereas the heterologous reaction with purified mouse globin mRNA did not exceed 10% hybridization. The $Rot_{1/2}$ of the homologous hybridization reaction was 6×10^{-4}, a value consistent with a complexity of 1250 nucleotides contributed by the α- plus β-globin sequences in our cDNA probe. Axel, R., et al., Cell 7: 247-254 (1976).

This rabbit globin cDNA was used as a probe in hybridization reactions with total RNA isolated from six transformed cell lines. Total RNA from transformed clone 6 protected 44% of the rabbit cDNA at completion, the value expected if only β-gene transcripts were present. This reaction displayed pseudo-first-order kinetics with $Rot_{1/2}$ of 2×10^3. A second transformant reacted with an $Rot_{1/2}$ of 8×10^3. No significant hybridization was observed at $Rots \geq 10^4$ with total RNA preparations from the four additional transformants.

We have characterized the RNA from clone 6 in greatest detail. RNA from this transformant was fractionated into nuclear and cytoplasmic populations to determine the intracellular localization of the rabbit globin RNA. The cytoplasmic RNA was further fractionated by oligo (dT)-cellulose chromatography into poly (A)⁺ and poly (A)⁻ RNA. Poly (A)⁺ cytoplasmic RNA from clone 6 hybridizes with the rabbit cDNA with an $Rot_{1/2}$ of 25. This value is 1/80th of the $Rot_{1/2}$ observed with total cellular RNA, consistent with the observation that poly (A)⁺ cytoplasmic RNA is 1-2% of the total RNA in a mouse cel. Hybridization is not detectable with either nuclear RNA or cytoplasmic poly (A)⁻ RNA at Rot values of 1×10^4 and 2×10^4, respectively. The steady-state concentration of rabbit β-globin RNA present in our transformant can be calculated from the $Rot_{1/2}$ to be about five copies per cell, with greater than 90% localized in the cytoplasm.

Several independent experiments argue that the globin RNA detected derives from transcription of the rabbit DNA sequences present in this transformant: (i) cDNA was prepared from purified 9S mouse globin RNA. This cDNA does not hybridize with poly (A)⁺ RNA from clone 6 at Rot values at which the reaction with rabbit globin cDNA is complete (ii) Rabbit globin cDNA does not hybridize with total cellular RNA obtained with tk⁺ globin⁻ transformants at Rot values exceding 10⁴. (iii) The hybridization observed does not result from duplex formation with rabbit globin DNA possibly contaminating the RNA preparations. Rabbit cDNA was annealed with total cellular RNA from clone 6, the reaction product was treated with S1 nuclease, and the duplex was subjected to equilibrium density centrifugation in cesium sulfate under conditions that separate DNA-RNA hybrids from duplex DNA. The S1-resistant cDNA banded at a density of 1.54 g/ml, as expected for DNA-RNA hybrid structures. These data, along with the observation that globin RNA is

polyadenylated, demonstrate that the hybridization observed with RNA preparations does not resul* from contaminating DNA sequences.

CHARACTERIZATION OF RABBIT GLOBIN TRANSCRIPTS IN TRANSFORMED CELLS

In rabbit erythroblast nuclei, the β-globin gene sequences are detected as a 14S precursor RNA that reflects transcription of two intervening sequences that are subsequently removed from this molecule to generate a 9S messenger RNA It was therefore of interest to determine whether the globin transcripts detected exist at a discrete 9S species, which is likely to reflect appropriate splicing of the rabbit gene transcript by the mouse fibroblast. Cytoplasmic poly (A)-containing RNA from clone 6 was electrophoresed on a methyl-mercury/agarose gel, Bailey, J. & Davidson, N., Anal. Biochem. 70: 75–85 (1976), and transferred to diazotized cellulose paper. Alwine, J. C. et al., Proc. Natl. Acad. Sci USA 74, 5340–5454 (1977). After transfer, the RNA on the filters was hybridized with DNA from the plasmid pβG1, which contains rabbit β-globin cDNA sequences. Maniatis, T., et al., Cell 8: 163–182 (1976). Using this ^{32}P-labeled probe, a discrete 9S species of RNA was observed in the cytoplasm of the transformant, which comigrated with rabbit globin mRNA isolated from rabbit erythroblasts. Hybridization to 9S RNA species was not observed in parallel lanes containing either purified mouse 9S globin RNA or poly (A)-containing cytoplasmic RNA from a tk+ transformant containing no rabbit globin genes.

In these experiments, it was not possible to detect the presence of a 14S precursor in nuclear RNA populations from the transformants. This is not surprising, because the levels expected in nuclear RNA, given the observed cytoplasmic concentration, are likely to be below the limits of detection of this techniques. The 5' and 3' boundaries of the rabbit globin sequences expressed in tranformed fibroblasts along with the internal processing sites can be defined more accurately by hybridizing this RNA with cloned DNAs, followed by S1 nuclease digestion and subsequent gel analysis of the DNA products. Berk, A. J. & Sharp, P. A., Cell 12: 721–732 (1977). When β-globin mRNA from rabbit erythroid cells was hybridized with cDNA clone p βG1 under appropriate conditions, the entire 576-base pair insert of cDNA was protected from S1 nuclease attack. When the cDNA clone was hybridized with RNA from our transformant, surprisingly, a discrete DNA band was observed at 525 base pairs, but not at 576 base pairs. These results suggest that, in this transformant, rabbit globin RNA molecules are present that have a deletion in a portion of the globin mRNA sequence at the 5' or 3' termini. To distinguish between these possibilities, DNA of the λ clone, RβG1, containing the chromosomal rabbit β-globin sequence hybridized with transformed fibroblast RNA. The hybrid formed was treated with S1 nuclease, and the protected DNA fragments were analyzed by alkaline agarose gel electrophoresis and identified by Southern blotting procedures. Southern, E. M., J. Mol. Biol. 98: 503–517 (1975). Because the rabbit β-globin gene is interrupted by two intervening sequences, the hybridization of mature rabbit mRNA to RβG1 DNA generates three DNA fragments in this sort of analysis: a 146-base pair fragment spanning the 5' terminus to the junction of the small intervening sequence, a 222-base pair internal fragment bridging the small and large intervening sequences, and a 221-base

pair fragment spanning the 3' junction of the large intervening sequence of the 3' terminus of the mRNA molecule. When transformant RNA was analyzed in this fashion, a 222-base pair fragment was observed as well as an aberrant fragment of 100 base pairs but no 146-base pair fragment. Hybridization with a specific 5' probe showed that the internal 222 base pair fragment was present. The sum of the protected lengths equaled the length of the DNA fragment protected by using the cDNA clone. Taken together, these results indicate that although the intervening sequences expressed in transformed mouse fibroblast are removed from the RNA transcripts precisely, the 5' termini of the cytoplasmic transcripts observed do not contain about 48 ± 5 nucleotides present in mature 9S RNA of rabbit erythroblasts.

DISCUSSION

In these studies, mouse cell lines have been constructed that contain the rabbit β-globin gene The ability of the mouse fibroblast recipient to transcribe and process this heterologous gene has then been analyzed. Solution hydbridization experiments in concert with RNA blotting techniques indicate that, in at least one transformed cell line, rabbit globin sequences are expressed in the cytoplasm as a polyadenylylated 9S species. Correct processing of the rabbit β-globin gene has also been observed in tk+ mouse cell transformants in which the globin and tk plasmids have been ligated prior to transformation. Mantei, N., et al., Nature (London) 281: 40–46 (1970). Similar results have been obtained by using a viral vector to introduce the rabbit globin gene into monkey cells. Hamer, D. H. & Leder, P., Nature (London), 281: 35–39 (1979); Mulligan, R. C., et al., Nature (London) 277: 108–114 (1979). Taken together, these results suggest that nonerythroid cells from heterologous species contain the enzymes necessary to correctly process the intervening sequences of a rabbit gene whose expression usually is restricted to erythroid cells.

The level of expression of rabbit globin sequences in the transformant is low: five copies of globin RNA are present in the cytoplasm of each cell. The results indicate that the two intervening sequences present in the original globin transcript are processed and removed at loci indistinguishable from those observed in rabbit erythroid cells. Surprisingly, 45 nucleotides present at the 5' terminus of mature rabbit mRNA are absent from the β-globin RNA sequence detected in the cytoplasm of the transformant examined. It is possible that incorrect initiation of transcription occurs about the globin gene in this mouse cell lines. Alternatively, the globin sequences detected may result from transcription of a long precursor that ultimately must undergo 5' processing to generate the mature 9S species. Incorrect processing at the 5' terminus in the mouse fibroblast could be responsible for the results. At present, it is difficult to distinguish among these alternatives. Because the analysis is restricted to a single transformant, it is not known whether these observations are common to all transformants expressing the globin gene or reflect a rare, but interesting abberation. It should be noted, however, that in similar experiments by Weissman and his colleagues, Mantei, N., et al., Nature (London) 281: 40–46 (1979), at least a portion of the rabbit globin RNA molecules transcribed in transformed mouse fibroblasts retain the correct 5' terminus.

Several alternative explanations can be offered for the expression of globin sequences in transformed fibro-

blasts. It is possible that constitutive synthesis of globin RNA occurs in cultured fibroblasts, Humphries, S., et al., Cell 7: 267–277 (1976), at levels five to six orders of magnitude below the level observed in erythroblasts. The introduction of 20 additional globin DNA templates may simply increase this constitutive transcription to the levels observed in the transformant. Alternatively, it is possible that the homologous globin gene is repressed by factors that are partially overcome by a gene dosage effect provided by the introduction of 20 additional globin genes. Finally, normal repression of the globin gene in a fibroblast may depend upon the position of these sequences in the chromosome. At least some of the newly introduced genes are likely to reside at loci distant from the resident mouse globin genes. Some of these ectopic sites may support low level transcription. Present data do not permit one to distinguish among these and other alternatives.

Although the number of rabbit globin genes within a given transformant remains stable for over a hundred generations of culture in hypoxanthine/aminopterin/-thymidine, it has not been possible to prove that these sequences are covalently integrated into recipient cell DNA. In previous studies, however, it has been demonstrated that cotransformation of either ΦX174 or plasmid pBR322 results in the stable integration of these sequences into high molecular nuclear DNA. In the present study, the globin gene represents a small internal segment of the high molecular weight concatenated phage DNA used in the transformation. Analysis of integration sites covalently linked to donor DNA is therefore difficult. Preliminary studies using radioactive λ sequences as a probe in DNA blotting experiments indicate that, in some cell lines, a contiguous stretch of recombinant phage DNA with a minimum length of 50 kbp has been introduced.

The presence of 9S globin RNA in the cytoplasm of transformants suggests that this RNA may be translated to give rabbit β-globin polypeptide. Attempts to detect this protein in cell lysates using a purified anti-rabbit β-globin antibody have thus far been unsuccessful. It is possible that the globin RNAs in the transformant are not translated or are translated with very low efficiency due to the absence of a functional ribosomal binding site. The cytoplasmic globin transcripts in the transformant lack about 48 nucleotides of untranslated 5' sequence, which includes 13 nucleotides known to interact with the 40S ribosomal subunit in nuclease protection studies. Efstratiadis, A., et al., Cell 10: 571–585 (1977); Legon, S., J. Mol. Biol. 106: 37–53 (1976). Even if translation did occur with normal efficiency, it is probable that the protein would exist at levels below the limits of detection of the immunologic assay due to the low level of globin RNA, and the observation that the half-life of β-globin in the absence of heme and globin may be less than 30 min. Mulligan, R. C., et al., Nature (London) 277: 108–114 (1979).

These studies indicate the potential value of cotransformation systems in the analysis of eucaryotic gene expression. The introduction of wild-type genes along with native and in vitro-constructed mutant genes into cultured cells provides an assay for the functional significance of sequence organization. It is obvious from these studies that this analysis will be facilitated by the ability to extend the generality of cotransformation to recipient cell lines, such as murine erythroleukemia cells, that provide a more appropriate environment for the study of heterologous globin gene expression.

THIRD SERIES OF EXPERIMENTS

The cotransformation experiments involving transformation of mouse cells with rabbit β-globin and with plasmid pBR322 and ΦX-174 DNA were continued and extended with the following results.

ΦX DNA was used in cotransformation experiments with the tk gene as the selectable marker. ΦX replicative from DNA was cleaved with Pst I, which recognizes a single site in the circular genome, Sanger. F et al., Nature 265: 687–695 (1977). Purified tk gene (500 pg) was mixed with 1–10 μg of Pst-cleaved ΦX replicative form DNA. This DNA was then added to mouse Ltk⁻ cells using the transformation conditions described herein and in Wigler, M., et al., Cell 16:777–785 (1979). After two weeks in selective medium (HAT), tk⁺ transformants were observed at a frequency of one colony per 10⁶ cells per 20 pg of purified gene. Clones were picked and grown into mass culture.

It was then asked whether tk³⁰ transformants contained ΦX DNA sequences. High molecular weight DNA from the transformants was cleaved with the restriction endonuclease Eco RI, which recognizes no sites in the ΦX genome. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nick-translated ³²P-ΦX DNA (blot hybridization).

These annealing experiments indicated that 15 to 16 transformants acquired bacteriophage sequences. Since the ΦX genome is not cut with the enzyme Eco RI, the number of bands observed reflects the minimum number of eucaryotic DNA fragments containing information homologous to ΦX. The clones contain variable amounts of ΦX sequences: 4 of the 15 positive clones reveal only a single annealing fragment while others reveal at least fifty ΦX-specific fragments.

It should be noted that none of 15 clones picked at random from neutral medium, following exposure to tk and ΦX DNA, contain ΦX information. Transformation with ΦX therefore is restricted to a subpopulation of tk⁺ transformants. The addition of a selectable marker therefore facilitates the identification of cotransformants.

TRANSFORMATION OF MOUSE CELLS WITH THE RABBIT β-GLOBIN GENE

Transformation with purified eucaryotic genes provides a means for studying the expression of cloned genes in a heterologous host. Cotransformation experiments were performed with the rabbit β major globin gene which was isolated from a cloned library of rabbit chromosomal DNA. One β-globin clone, designated R G-1 consists of a 15 kb rabbit DNA fragment carried on the bacteriophage λ cloning vector Charon 4A. Intact DNA from this clone (RβG-1) was mixed with the viral tk DNA at a molar ratio of 100:1, and tk⁺ transformants were isolated and examined for the presence of rabbit globin sequences. Cleavage of RβG-1 with the enzyme Kpn I generates a 4.7 kb fragment which contains the entire rabbit β-globin gene. This fragment was purified by gel electrophoresis and nick-translated to generate a probe for subsequent annealing experiments. The β-globin genes of mouse and rabbit are partially homologous, although we do not observe annealing of the rabbit β-globin probe with Kpn-cleaved mouse DNA, presumably because Kpn generates very large globin-specific fragments. In contrast, cleavage of rabbit liver DNA with Kpn I generates the expected 4.7 kb globin

band. Cleavage of transformed cell DNA with the enzyme Kpn I generates a 4.7 kb fragment containing globin-specific information in six of the eight tk+ transformants examined. The number of rabbit globin genes present in these transformants is variable. In comparison with controls, some of the clones contain a single copy of the gene, while others may contain as many as 20 copies of this heterologous gene.

RABBIT β-GLOBIN SEQUENCES ARE TRANSCRIBED IN MOUSE TRANSFORMANTS

The cotransformation system developed provides a functional assay for cloned eucaryotic genes if these genes are expressed in the heterologous recipient cell. Six transformed cell clones were analyzed for the presence of rabbit β-globin RNA sequences. In initial experiments, solution hybridization reactions were performed to determine the cellular concentration of rabbit globin transcripts in transformants.

A radioactive cDNA copy of purified rabbit α and β-globin mRNA was annealed with a vast excess of total cellular RNA from transformants under experimental conditions such that rabbit globin cDNA does not form a stable hybrid with mouse sequences. Total RNA from transformed clone 6 protects 44% of the rabbit cDNA at completion, the value expected if only β gene transcripts are present. This reaction displays pseudo-first-order kinetics with an $R_oT_{1/2}$ of 2×10^3. A second transformant (clone 2) reacts with an $R_oT_{1/2}$ of 8×10^3. No significant hybridization was observed with total RNA preparations from four other transformants. Further analysis of clone 6 demonstrates that virtually all of the rabbit β-globin RNA detected in this transformant is polyadenylated and exists at a steady state concentration of about five copies per cell with greater than 90% of the sequences localized in the cytoplasm.

GLOBIN SEQUENCES EXIST AS A DISCRETE 9S SPECIES IN TRANSFORMED CELLS

In rabbit erythroblast nuclei, the β-globin gene sequences are detected as a 14S precursor RNA which reflects transcription of two intervening sequences which are subsequently spliced from this molecule to generate a 9S messenger RNA. Our solution hybridization experiments only indicate that polyadenylated rabbit globin RNA sequences are present in the mouse transformant. It was therefore of interest to determine whether the globin transcripts we detected exist as a discrete 9S species, which is likely to reflect appropriate splicing of the rabbit gene transcript by the mouse fibroblast. Cytoplasmic poly A-containing RNA from clone 6 was denatured by treatment with 6M urea at 70° C., and electrophoresed on a 1% acid-urea-agarose gel and transferred to diazotized cellulose paper. Following transfer, the RNA filters were hybridized with DNA from the plasmid RβG-1 containing rabbit β-globin cDNA sequences. Using this ³²P-labeled probe, a discrete 9S species of cytoplasmic RNA is seen which co-migrates with rabbit globin mRNA isolated from rabbit erythroblasts. Hybridization to 9S RNA species is not observed in parallel lanes containing either purified mouse 9S globin RNA or polyadenylated cytoplasmic RNA from a tk+ transformant containing no rabbit globin genes.

One is unable in these experiments to detect the presence of a 14S precursor in nuclear RNA populations from the transformant. This is not surprising, since the levels expected in nuclear RNA, given the observed

cytoplasmic concentration, are likely to be below the limits of detection of this technique. Nevertheless, the results with cytoplasmic RNA strongly suggest that the mouse fibroblast is capable of processing a transcript of the rabbit β-globin gene to generate a 9S polyadenylated species which is indistinguishable from the β-globin mRNA in rabbit erythroblasts.

RESCUE OF pBR 322 DNA FROM TRANSFORMED MOUSE CELLS

Observations on cotransformation were extended to the Ek-2 approved bacterial vector, plasmid pBR 322. Using the cotransformation scheme outlined herein, cell lines were constructed containing multiple copies of the pBR 322 genome. Blot hybridization analyses indicate that the pBR 322 sequences integrate into cellular DNA without significant loss of plasmid DNA. pBR 322 DNA linearized with either Hind III or Bam HI, which destroys the tetracycline resistance gene, integrates into mouse DNA with retention of both the plasmid replication origin and the ampicillin resistance (β-lactamase) gene. It was therefore asked whether these plasmid sequences could be rescued from the mouse genome by a second transformation of bacterial cells.

The experimental approach chosen is outlined in FIG. 2. Linearized pBR 322 DNA is introduced into mouse Ltk⁻ cells via cotransformation using the tk gene as a selectable marker. DNA is isolated from transformants and screened for the presence of pBR 322 sequences. Since the donor plasmid is linearized, interrupting the tetracycline resistant gene, transformed cell DNA contains a linear stretch of plasmid DNA consisting of the replication origin and the β-lactamase gene covalently linked to mouse cellular DNA. This DNA is cleaved with an enzyme such as Xho I, which does not digest the plasmid genome. The resulting fragments are circularized at low DNA concentrations in the presence of ligase. Circular molecules containing plasmid DNA are selected from the vast excess of eucaryotic circles by transformation of E. coli strain χ1776.

This series of experiments has been carried out and a recombinant plasmid isolated from transformed mouse cell DNA which displays the following properties: (1) The rescued plasmid is ampicillin resistant, but tetracycline sensitive consistent with the fact that the donor pBR 322 was linearized by cleavage within the tetracycline resistance gene. (2) The rescue plasmid is 1.9 kb larger than pBR 322 and therefore contains additional DNA. (3) The rescued plasmid anneals to a single band in blot hybridizations to Eco RI-cleaved mouse liver DNA, suggesting that the plasmid contains an insert of single copy mouse DNA. These observations demonstrate that bacterial plasmids stably integrated into the mouse genome via transformation, can be rescued from this unnatural environment, and retain their ability to function in bacterial hosts.

This result immediately suggests modified schemes utilizing plasmid rescue to isolate virtually any cellular gene for which selective growth criteria are available. The aprt gene of the chicken is not cleaved by Hind III or Xho I and transformation of aprt⁻ mouse cells with cellular DNA digested with these enzymes results in the generation of aprt⁺ colonies which express the chicken aprt gene. Ligation of Hind III cleaved chicken DNA with Hind III cleaved pBR 322 results in the formation of hybrid DNA molecules, in which the aprt gene is now adjacent to plasmid sequences. Transformation of aprt⁻ cells is now performed with this DNA. Transfor-

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mants should contain the aprt gene covalently linked to pBR 322, integrated into the mouse genome. This transformed cell DNA is now treated with an enzyme which does not cleave either pBR 322 or the aprt gene, and the resultant fragments are circularized with ligase. Transformation of *E. coli* with these circular molecules should select for plasmid sequences from eucaryotic DNA and enormously enrich for chicken aprt sequences. This double selection technique permits the isolation of genes expressed at low levels in eucaryotic cells, for which hybridization probes are not readily obtained.

DISCUSSION

The frequency with which DNA is stably introduced into competent cells is high. Furthermore, the cotransformed sequences appear to be integrated into high molecular weight nuclear DNA. The number of integration events varies from one to greater than fifty in independent transformed clones. At present, precise statements cannot be made concerning the nature of the integration intermediate. Although data with ΦX are in accord with the model in which ΦX DNA integrates as a linear molecule, it is possible that more complex intramolecular recombination events generating circular intermediates may have occurred prior to or during the integration process. Whatever the mode of integration, it appears that cells can be stably transformed with long stretches of donor DNA. It has been observed that transformants contain continguous stretches of donor DNA 50 kb long. Furthermore, the frequency of competent cells in culture is also high. At least one percent of the mouse Ltk⁻ cell recipients can be transformed to the tk⁺ phenotype. Although the frequency of transformation in nature is not known, this process could have profound physiologic and evolutionary consequences.

The introduction of cloned eucaryotic genes into animal cells provides an in vivo system to study the functional significance of various features of DNA sequence organization. In these studies, stable mouse cell lines have been constructed which contain up to 20 copies of the rabbit β-globin gene. The ability of the mouse fibroblast recipient to transcribe and process this heterologous gene has been analyzed. Solution hybridization experiments in concert with RNA blotting techniques indicate that in at least one transformed cell line, rabbit globin sequences are expressed in the cytoplasm as a 9S species indistinguishable from the mature messenger RNA of rabbit erythroblasts. These results suggest that the mouse fibroblast contains the enzymes necessary to transcribe and correctly process a rabbit gene whose expression is normally restricted to erythroid cells. Similar observations have been made by others using a viral vector to introduce the rabbit globin gene into monkey cells.

These studies indicate the potential value of cotransformation systems in the analysis of eucaryotic gene expression. The introduction of wild type genes along with native and in vitro constructed mutant genes into cultured cells provides an assay for the functional significance of sequence organization. It is obvious from these studies that this analysis will be facilitated by the ability to extend the generality of cotransformation to recipient cell lines, such as murine erythroleukemia cells, which may provide a more appropriate environment for the study of heterologous globin gene expression.

FOURTH SERIES OF EXPERIMENTS

The ability to transfer purified genes into cultured cells provides the unique opportunity to study the function and physical state of exogenous genes in the transformed host. The development of a system for DNA-mediated transfer of the HSV thymidine kinase (tk) gene to mutant mouse cells, Wigler, M., et al., Cell 11:223-232 (1977), has permitted extension of these studies to unique cellular genes. Wigler, M., et al., Cell 14:725-731 (1979). It has been found that high molecular weight DNA obtained from tk⁺ tissues and cultured cells from a variety of eucaryotic organisms can be used to transfer tk activity to mutant mouse cells deficient in this enzyme. The generality of the transformation process has been demonetrated by the successful transfer of the cellular adenine phosphoribosyl transferase (aprt) gene and the hypoxanthine phosphoribosyl transferase (hprt) gene. Wigler, M., et al., Proc. Nat. Acad. Sci. USA 76:1373-1376 (1979); Willicke, K., et al., Molec. Gen. Genet. 170:179-185 (1979); Graf, L. Y., et al, Somatic Cell Genetics, in press (1979).

More recently, it has been demonstrated that cells transformed with genes coding for selectable biochemical markers also integrate other physically unlinked DNA fragments at high frequency. In this manner, the tk gene has been used as a marker to identify mammalian cells cotransformed with defined procaryotic and eucaryotic genes into cultured mammalian cells. Wigler, M., et al., Cell 16:777-785 (1979).

Detection of gene transfer has in the past relied extensively on the use of appropriate mutant cell lines. In some cases, cells resistant to metabolic inhibitors contain dominant acting mutant genes. Cotransformation with such dominant acting markers should in principle permit the introduction of virtually any cloned genetic element into wild type cultured cells. In this study, cells were transformed with the gene coding for a mutant dihydrofolate reductase (dhfr) gene which renders cells resistant to high concentrations of methotrexate (mtx). Flintoff, W. F., et al., Cell 2:245-262 (1976).

Cultured mammalian cells are exquisitely sensitive to the folate antagonist, methotrexate. Mtx resistant cell lines have been identified which fall into three categories: (1) cells with decreased transport of this drug. Fischer, G. A. Biochem. Pharmacol. 11:1233-1237 (1962); Sirotnak, F. M., et al., Cancer Res. 28 75-80 (1968); (2) cells with structural mutations which lower the affinity of dhfr for methotrexate. Flintoff, W F, et al., Cell 2:245-262 (1976); and (3) cells which produce inordinately high levels of dhfr. Biedler, J. L., et al., Cancer Res. 32: 152-161·(1972); Chang, S. E., and Littlefield, J. W., Cell 7:391-396 (1976). Where they have been examined, cells producing high levels of dhfr have been found to contain elevated levels of the dhfr gene (gene amplification). Schimke, R. T., et al, Science 202:1051-1055 (1978).

An interesting methotrexate resistant variant cell line (A29) has been identified that synthesizes elevated levels of a mutant dihydrofolate reductase with reduced affinity for methotrexate. Wigler, M., et al. Cell 16:777-785 (1979). Genomic DNA from this cell line has been used as donor in experiments to transfer the mutant dhfr gene to mtx sensitive cells. Exposure of mtx resistant transformed cells to increasing levels of mtx selects for cells which have amplified the transferred gene. In this way, it is possible to transfer and amplify

virtually any genetic element in cultured mammalian cells.

TRANSFER OF THE MUTANT HAMSTER DIHYDROFOLATE REDUCTASE GENE TO MOUSE CELLS

High molecular weight cellular DNA was prepared from wildtype mtx sensitive CHO cells and from A29 cells, an mtx resistant CHO derivative synthesizing increased levels of a mutant dhfr. Flintoff, W. F., et al., Cell 2:245–262 (1976). The ability of these DNA preparations to transfer either the dhfr gene or the tk gene to tk− mouse L cells (Ltk− aprt−) was tested using a modification of the calcium phosphate coprecipitation method. Wigler, M., et al., Proc. Nat. Acad. Sci. USA 76.1373–1376 (1979). DNA from both mutant A29 and wild-type CHO cells was competent in transferring the tk gene to Ltk− aprt− cells. Methotrexate resistant colonies were observed only following treatment of cells with DNA from A29. The data obtained suggest that treatment of methotrexate sensitive cells with A29 DNA resulted in the transfer and expression of a mutant dhfr gene, thus rendering these cells insensitive to elevated levels of methotrexate.

In order to test this hypothesis directly, molecular hybridization studies were performed to demonstrate the presence of the hamster dhfr gene in DNA from presumed transformants. A mouse dhfr cDNA clone (pdfr-21), Chang, A.C.Y., et al., Nature 275:617–624 (1978), that shares homology with the structural gene sequences of the hamster dhfr gene was used to detect the presence of this gene in our transformants. Restriction analysis of the dhfr gene from A29, from presumed transformants, and from amplified mouse cells, was performed by blot hybridization. Southern, E. M., J. Mol. Biol. 98:503–517 (1975). DNA was cleaved with restriction endonuclease Hind III, electrophoresed in agarose gels, and transferred to nitrocellulose filters. These fileres were then hybridized with high specific activity, ^{32}P-labeled nick-translated pdhfr-21 and developed by autoradiography. This procedure visualizes restriction fragments of genomic DNA homologous to the dhfr probe. Prominent bands are observed at 15 kb, 3.5 kb and 3 kb for mouse DNA and 17 kb, 7.9 kb, 3.7 kb and 1.4 kb for hamster DNA. The restriction profiles between these two species are sufficiently different to permit one to distinguish the hamster gene in the presence of an endogenous mouse gene. Five L cell transformants resistant to methotrexate were therefore examined by blot hybridization. In each transformed cell line, one observed the expected profile of bands resulting from cleavage of the endogenous mouse dhfr gene and a series of additional bands whose molecular weights are identical to those observed upon cleavage of hamster DNA. The 17.9 kb, 7.9 kb and 1.4 kb bands observed in hamster DNA are diagostic for the presence of the hamster dhfr gene and are present in all transformants.

In initial experiments, the lowest concentration of methotrexate (0.1 μg per ml) was chosen which would decrease survival of Ltk− aprt− cells to less than 10^{-7}. Previous studies, Flintoff, W. F., et al., Cell 2:245–262 (1976), suggested that the presence of a single mutant dhfr gene can render cells resistant to this concentration of methotrexate. Comparison of the intensity of the hamster dhfr gene fragments of transformed cell DNA with those of wild-type hamster DNA suggest that our transformants contain one or at most a few methotrex-

ate resistant hamster genes. By contrast, donor A29 cells, which have been shown to produce elevated levels of the mutant dhfr, Flintoff, W. F., et al. Cell 2.245–262 (1976), appear to contain multiple copies of this gene.

AMPLIFICATION OF THE TRANSFERRED dhfr GENE

Initial transformants were selected for resistance to relatively low levels of mtx (0.1 μg/ml). For every clone, however, it was possible to select cells resistant to elevated levels of mtx by exposing mass cultures to successively increasing concentrations of this drug. In this manner, we isolated cultures resistant to up to 40 μg/ml of methotrexate starting from clones that were initially resistant to 0.1 μg/ml. We next asked if increased resistance to methtrexate in these transformants was associated with amplification of a dhfr gene and, if so, whether the endogenous mouse or the newly transferred hamster gene was amplified. DNA from four independent isolates and their resistant derivatives was examined by blot hybridization. In each instance, enhanced resistance to methotrexate was accompanied by an increase in the copy number of the hamster gene. This is most readily seen by comparing the intensities of the 1.5 kb band. In no instance have we detected amplification of the endogenous mouse dhfr gene. Lastly, it is noted that not all lines selected at equivalent methotrexate concentrations appear to have the same dhfr gene copy number.

THE dhfr GENE AS A GENERALIZED TRANSFORMATION VECTOR

Selectable genes can be used as vectors for the introduction of other genetic elements into cultured cells. In previous studies, it has been demonstrated that cells transformed with the tk gene are likely to incorporate other unlinked genes. Wigler, M., et al., Cell 16:777–785 (1979). The generality of this approach was tested for the selectable marker, the mutant dhfr gene. 20 μg of total cellular DNA from A29 was mixed with 1 μg of Hind III-linearized pBR 322 DNA. Recipient cells were exposed to this DNA mixture and, after two weeks, methotrexate resistant colonies were picked. Genomic DNA from transformants was isolated, cleaved with Hind III and analyzed for the presence of pBR322 sequences. Two independent isolates were examined in this way and in both cases multiple copies of pBR322 sequences were present in these methotrexate transformants.

An alternate approach to generalized transformation involves ligation of a nonselectable DNA sequence to a selectable gene. Since the muant dhfr gene is a dominant acting drug resistance factor, this gene is an ideal vector. Furthermore, it should be possible to amplify any genetic element ligated to this vector by selecting cells resistant to elevated levels of mtx. To explore this possibility, restriction endonucleases that do not destroy the dhfr gene of A29 were identified by transformation assay. One such restriction endonuclease, Sal I, does not destroy the transformation potential of A29 DNA. Sal I-cleaved A29 DNA was therefore ligated to an equal mass of Sal I-linearized pBR32. This ligation product was subsequently used in transformation experiments. Methotrexate resistant colonies were picked and grown into mass culture at 0.1 μg methotrexate/ml. Mass cultures were subsequently exposed to increasing concentrations of methotrexate.

DNAs were obtained from mass cultures resistant to 0.1, 2, 10 and 40 μg/ml methotrexate, and the copy number of pBR322 and dhfr sequences was determined by blot hybridization. Six independent transformed lines were examined in this fashion. Five of these lines exhibited multiple bands homologous to pBR322 sequences. In four of these transformed clones, at least one of the pBR 322-specific bands increased in intensity upon amplification of dhfr. In SS-1, two pBR322-specific bands are observed in DNA from cells resistant to 0.1 μg/ml methotrexate. These bands increase several-fold in intensity in cells resistant to 2 μg/ml. No further increase in intensity is observed, however, in cells selected for resistance to 40 μg/ml/ In a second line, SS-6, all pBR 322 bands present at 0.1 μg/ml continue to increase in intensity as cells are selected first at 2 μg/ml and then at 40 μg/ml methotrexate. Curiously, new pBR322-specific bands appear after selection at higher methotrexate concentrations. It was estimated that there is at least a fifty-fold increase in copy number for pBR322 sequences in this cell line. In a third cell line, HH-1, two pBR322-specific bands increase in intensity upon amplification, others remain constant or decrease in intensity. Thus, the pattern of amplification of pBR322 sequences observed in these cells can be quite varied. Nevertheless, it appears that the mutant dhfr gene can be used as vector for the introduction and amplification of defined DNA sequences into cultured animal cells.

DISCUSSION

The potential usefulness of DNA-mediated transformation in the study of eucaryotic gene expression depends to a large extend on its generality. Cellular genes coding for selectable biochemical functions have previously been introduced into mutant cultured cells, Wigler, M., et al., Cell 14:725-731 (1979); Wigler, M., et al., Proc. Nat. Acad. Sci. USA 76:1373-1376 (1979); Wilecke, K., et al., Molec. Gen. Genet. 170:179-185 (1979); Graf, L. H., et al., Somatic Cell Genetics, in press (1979). In the present study, a dominant acting, methotrexate resistant dhfr gene has been transferred to wild-type cultured cells. The use of this gene as a vector in cotransformation systems may now permit the introduction of virtually any genetic element into a host of new cellular environments.

In initial experiments, DNA from A29 cells, a methotrexate resistant CHO derivative synthesizing a mutant dhfr was added to cultures of mouse L cells. Methotrexate resistant colonies appeared at a frequency of one to ten colonies/5 × 10⁵ cells/20 μg cellular DNA. No colonies were observed upon transformation with DNA obtained from wild-type, methotrexate sensitive cells, although this DNA was a competent donor of the thymidine kinase gene. Definitive evidence that we have effected transfer of a mutant hamster dhfr gene was obtained by demonstrating the presence of the hamster gene in mouse transformants. The restriction maps of the mouse and hamster dhfr genes are significantly different and permit one to distinguish these genes in blot hybridization experiments. In all transformants examined, one observes two sets of restriction fragments homologous to a mouse dhfr cDNA clone: a series of bands characteristic of the endogenous mouse gene and a second series characteristic of the donor hamster gene.

The utility of transformation of the dhfr locus is a function of the relative frequencies both of transformation and of spontaneous resistance to mtx. The demonstration that all mtx resistant L cells picked result from transformation rather than amplification of endogenous genes suggests that amplification of dhfr is a rare event in this cell line. Attempts were made to transform other cell lines, including mouse teratoma and rat liver cells and, in these instances, hybridization studies reveal that the acquisition of mtx resistance results from amplification of endogenous dhfr genes. The use of a purified dhfr gene is likely to overcome these difficulties by enormously increasing the frequency of transformation.

The dhfr copy number observed in initial transformants is low. This observation is consistent with previous studies suggesting that a single mutant dhfr gene is capable of rendering cells mtx resistant under selective criteria (0.1 μ/ml mtx). Flintoff, W. F., et al., Cell 2: 245-262 (1976). Exposure of these initial mtx resistant transformants to stepwise increases in drug concentration results in the selection of cells with enhanced mtx resistance resulting from amplification of the newly transferred mutant hamster dhfr gene. In no transformants has amplification of the endogenous mouse gene been observed in response to selective pressure. It is likely that a single mutant gene affords significantly greater resistance to a given concentration of mtx than a single wild-type gene. If the frequency of the amplification is low, one is merely selecting resistance variants having the minimum number of amplification events. It is also possible that newly transferred genes may be amplified more readily than endogenous genes.

The mutant dhfr gene has been used as a dominant transfer vector to introduce nonselectable genetic elements into cultured cells. One experimental approach exploits the observation made previously, Wigler, M., et al.; Cell 16: 777-785 (1979), that competent cells integrate other physically unlinked genes at high frequency. Cultures exposed to pBR322 DNA, along with the genomic DNA containing the mutant dhfr gene give rise to mtx resistant cell lines containing multiple copies of the bacterial plasmid.

An alternative approach to genetic vectoring involves ligation of pBR322 sequences to the selectable dhfr gene prior to transformations. This procedure also generates transformants containing multiple pBR322 sequences. Amplification of dhfr genes results in amplification of pBR322 sequences, but the patterns of amplification differ among cell lines. In one instance, all pBR322 sequences amplify with increasing mtx concentrations. In other lines, only a subset of the sequences amplify.

In yet other lines, sequences appear to have been lost or rearranged. In some lines, amplification proceeds with increasing mtx concentrations up to 40 μ/ml, whereas in others, amplification ceases at 2 μg/ml. At present, the amplification process is not understood nor has the amplification unit been defined. Whatever the mechanisms responsible for these complex events, it is apparent that they can be exploited to control the dosage of virtually any gene introduced into cultured cells.

FIFTH SERIES OF EXPERIMENTS

Mouse teratocarcinoma (TCC) stem cells provide a unique vector for the introduction of specific, predetermined, genetic changes into mice. Mintz, B. & Illmensee, K., Proc. Natl. Acad. Sci. 72: 3585-3589 (1975); Mintz, B., Brookhaven Symp. Biol. 29: 82-85 (1977). These cells lose their neoplastic properties and undergo normal differentiation when placed in the environment of the early embryo. There they can contribute to for-

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mation of all somatic tissues in a mosaic .. : . "pris-
ing both donor- and host-derived cells, a... s. to the
germ line, from which the progeny have gen... of the
tumor strain in all their cells. Thus, during initial propa-
gation of TCC stem cells in culture, clones with experi-
mentally selected nuclear, Dewey, M. J., et al., Proc.
Natl. Acad. Sci., 74: 5564–5568 (1977), and cytoplasmic,
Watanabe, T., et al., Proc. Natl. Acad. Sci., 75:
5113–5117 (1978), gene mutations have been obtained
and the cells have proved capable of participating in
embryogenesis.

The effective application of this system in probing the
control of gene expression during differentiation would
be greatly enhanced if, as proposed, Mintz, B., Differen-
tiation 13: 25–27 (1979), precisely defined genes, either
in native or modified form, with known associated se-
quences, could be introduced into developmentally
totipotent TCC cells prior to their development in vivo.
DNA-mediated gene transfer into cultured mouse cells
has now been reported for a variety of viral and cellular
genes coding for selectable biochemical functions. The
purified viral thymidine kinase (tk; ATP: thymidine
5'-phosphotransferase, EC 2.7.1.21) gene has provided a
model system for gene transfer, Wigler, M. et al., Cell
11: 223–232 (1977), and has been followed by the DNA-
mediated transfer of the cellular genes coding for thy-
midine kinase, Wigler, M., et al., Cell 14: 725–731
(1978), hypoxanthine phosphoribosyltransferase, Wil-
lecke, K., et al., Molec. Gen. Genet. 170: 179–185
(1979), Graf, L. H., et al., Somat. Cell Genet., in press
(1979), adenine phosphoribosyltransferase, Wigler, M.,
et al., Proc. Natl. Acad. Sci. USA, 76: 1373–1376 (1979),
and dihydrofolate reductase, Wigler, M., et al., Proc.
Natl. Acad. Sci., in press (1980); Lewis, W. H., et al.,
Somat. Cell. Genet., in press (1979). In this report is
demonstrated the contransfer of the cloned Herpes sim-
plex (HSV) thymidine kinase gene along with the
human β-globin gene into mutant (tk⁻) teratocar-
cinoma stem cells in culture. These transformed cells,
when tested by subcutaneous inoculation into mice,
retain their developmental capacities in the tumors that
are produced, and exhibit the viral-specific tk enzy-
matic activity for numerous cell generations in vivo.

TRANSFORMATION OF TK⁻
TERATOCARCINOMA CELLS

The addition of plasmid DNA containing the HSV
thymidine kinase gene to cultures of attached mouse L
tk⁻ cells yields L tk⁺ transformants in HAT at a fre-
quency of one colony per 100 pg of DNA per 5×10⁵
cells. Under identical transformation procedures, tk⁻
teratocarcinoma cells showed a strikingly lower trans-
formation efficiency. Based on the average of three
independent experiments, one surviving colony was
obtained per 4 μg of plasmid DNA per 5×10⁵ cells, a
value four to five orders of magnitude below that of the
L tk⁻ cells. This relatively low efficiency was con-
firmed when the DNA was added to TCC tk⁻ cells in
suspension. Addition of 10 μg of Bam H1-restricted
ptk-1 DNA to 7×10⁶ cells resulted in only four trans-
formants in HAT. With identical transformation condi-
tions, L tk⁻ cells gave 3×10³ tk⁺ colonies per 10⁷ cells
per 1.5 μg of ptk-1 DNA. While high concentrations of
gene are thus required to effect transformation in this
teratocarcinoma cell line, the availability of cloned
DNA nonetheless allows numerous tk⁺ transformants
to be obtained.

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EXPRESSION OF HSV TK ACTIVITY IN
TRANSFORMED TERATOCARCINOMA CELLS

To ascertain whether the tk⁺ phenotypes of the TCC
clones were indeed attributable to expression of the
viral tk gene, seven colonies were picked from indepen-
dent culture dishes and grown into mass cultures for
testing. The activity of five clones were characterized
by serological, and of two by biochemical, techniques.
The Herpes-type antigenic identity of tk was verified by
assaying the ability of HSV- tk-specific antibody to
neutralize enzymatic activity. Over 90% inhibition of tk
activity was in fact observed when immune serum was
reacted with extracts of each of the five transformed
clones chosen (Table I). The low residual activity re-
maining after neutralization of transformed-cell extracts
may represent mitochondrial tk activity, which by itself
is unable to afford survival in HAT. Cell extracts from
the other two TCC tk⁺ clones chosen were tested for tk
electrophoretic mobility because of the marked differ-
ence between the mouse and HSV tk enzymes. While
the TCC tk⁻ control, as expected, shows no major peak
of activity, the transformants have the HSV tk charac-
teristic peak migrating with an R/of 0.45, as shown for
one of the clones.

TABLE 1

Cell line source of extract	Specific neutralization of Herpes thymidine kinase in transformants		
	Activity with preimmune serum Units × 10⁻³ per ml	Activity with antiserum	
		Units × 10⁻³ per ml	% Residual activity
TCC wt*	2.8	3.0	107 0
TCC tk⁻**	0.05	0.06	100 0
LHB 2b*	34	0 06	2 0
TCC tk-1⁸	2.1	0.17	8 0
TCC tk-3	5.5	0 43	8.0
TCC tk-4	6.1	0.15	2.5
TCC tk-5	3 7	0 21	6 0

30,000 × g supernatants of homogenates (S-30) from the indicated cell lines were
mixed with preimmune serum or antiserum to purified HSV-1 tk, and tk activity was
assayed as described in Materials and Method. Activity is expressed as units per ml
of the S-30 fraction.
*TCC wt is a mouse teratocarcinoma feeder-independent cell line (6050P) with tk⁺
(wild-type) phenotype
**TCC tk⁻ is a derivative of TCC wt that is resistant to BrdUrd and is tk-deficient
*LHB 2b is a mouse L tk⁻ cell line transformed to the tk⁺ phenotype with the
Herpes thymidine kinase gene.
ᵇTCC tk-1, -3, -4, and -5 are HAT-resistant teratocarcinoma clones derived from
TCC tk⁻ after transformation with the Herpes thymidine kinase gene

THE PHYSICAL STATE OF THE TK GENE IN
TRANSFORMED TERATOCARCINOMA CELLS

The number of viral tk gene fragments and the loca-
tion of these fragments in independent transformants
were examined utilizing the blot hybridization tech-
nique of Southern, Southern, E. M., J. Mol. Biol., 98:
503–517 (1975). The donor DNA was the recombinant
plasmid, ptk-1, digested to completion with Bam H1.
This plasmid contains a 3.4 kb fragment with the viral tk
gene inserted at the single Bam H1 site within the tetra-
cycline resistance gene of pBR322. Transformation
with Bam-cleaved tk DNA results in integration with
loss of the Bam sites at the termini of the 3.4 kb frag-
ment. High molecular weight DNA from transformants
was cleaved with Bam H1, fractionated by agarose gel
electrophoresis, and transferred to nitrocellulose filters;
the filters were then annealed with nick-translated ³²P-
tk DNA. In each cell clone, a single annealing fragment
was seen; therefore, each clone contains at least one

viral tk gene. As expected, each clone reveals a band of molecular weight greater than 3.4 kb. The molecular weights of the annealing fragments diffe. among the transformed clones, a result suggesting that integration has occurred at different sites within the DNA of the respective transformants.

STABILITY OF THE TRANSFORMED PHENOTYPE IN CULTURE

To test the capacity of the TCC transformants to retain expression of the donor tk gene in culture in the absence of selective pressure, individual clones grown into mass culture in HAT selective medium were sub-cultured for various periods in the absence of the selec-tive agent. The fraction of cells that retained the tk+ phenotype was determined by measuring cloning effi-ciencies in selective and nonselective media. Wide dif-ferences among clones became apparent (Table II). Some cell lines,

TABLE II

In vitro stability of the transformed phenotype in teratocarcinoma cells			
Clonal cell line	Experi-ment	Generations in nonselective medium*	Relative cloning efficiency in selective medium**	Rate of loss of tk+ phenotype per generation#
TCC tk-1	1	28	0.45	
	2	150	0.50	<0.001
TCC tk-2	1	28	0.23	
	2	150	0.02	0.017
TCC tk-3	1	28	0.47	
	2	150	0.27	0.002
TCC tk-4	1	28	0.26	
	2	150	0.16	0.003
TCC tk-5	1	28	0.14	
	2	150	0.01	0.021

*Clones were picked and grown in HAT selective medium for 40 cell generations. Cells were then grown in nonselective medium for 28 or 150 generations prior to determining their cloning efficiencies under selective and nonselective conditions.
**One hundred cells were plated in triplicate into HAT selective and nonselective media. The relative cloning efficiency in selective medium is defined as the ratio of the cloning efficiency under selective conditions to the cloning efficiency under nonselective conditions (50-70%).
#In these calculations it is assumed that for any given cell line the rate of loss of the tk phenotype is constant in each cell generation. The rate of loss per generation may then be calculated from the formula $F_M (1 - X)^{N-M} + F_N$ in which F_M is the relative cloning efficiency in selective medium after M generations in nonselective medium. F_N is similarly defined for N generations, and X is the rate of loss per cell generation, such as TCC tk-1, were relatively stable and lost the tk+ phenotype at frequencies less than 0.1% per generation in nonselective medium. Other, less stable lines (TCC tk-2 and TCC tk-5) lost tk+ expression at 2% per generation in the absence of selection.

MAINTENANCE AND EXPRESSION OF THE HSV TK GENE IN VIVO DURING TISSUE DIFFERENTIATION IN TUMORS

The more critical question of retention of the foreign gene and of its expression during TCC cell differentia-tion in vivo in the absence of selection was examined in solid tumors. Tumors were formed by inoculating syn-geneic hosts (usually two hosts per clone) subcutane-ously with 10^7 cells from each of the same five trans-formed clones. DNA from these tumors was analyzed by blot hybridization. Neutralization assays and electro-phoretic mobility tests of the tk enzyme were also car-ried out to identify expression of the viral gene. In addi-tion, samples of the same tumors were fixed and exam-ined histologically for evidence of differentiation.

The restriction fragment profiles of the viral tk gene demonstrated that the gene was retained in all nine tumors-analyzed. When each tumor (grown without HAT selection) was compared with its cell line of ori-gin (cultured under HAT selective pressure), the num-

ber and location of the annealing fragments in seven of the tumors was identical to that of the corresponding cell line. Thus, the introduced tk gene was, in most cases, maintained for many cell generations spanning at least three weeks in vivo without significant loss or translocation. In two instances, however, a gene rear-rangement had occurred, resulting from the loss of the original tk-containing fragment and the appearance of a new fragment of different molecular weight. It is of interest that these two tumors were produced from the two TCC clones that lost the tk+ phenotype in vitro at highest frequencies (Table II).

The results of neutralization tests with HSV-tk-specific antiserum demonstrated that at least three of the nine tumors (including one from the TCC tk-1 clone) had viral-type tk activity. (The presence of host cells in the tumors probably contributed substantial amounts of non-neutralized mouse tk in the remaining cases.) Another sample of the tumor derived from the TCC tk-1 line was also analyzed electrophoretically for HSV tk activity; a predominant peak migrating with an R_f of 0.45, characteristic of the viral enzyme, was ob-served.

Histological speciments from each of the tumors were prepared and examined. In addition ot the TCC stem cells, tumors contained an array of differentiated tissues similar to those in tumors from the untrans-formed TCC wt and TCC tk- cell lines of origin. In-cluded were muscle, neural formations, adipose tissue, some bone, squamous keratinizing epithelium, and other epithelia, ducts, and tubules.

COTRANSFORMATION OF TERATOCARCINOMA CELLS WITH THE HUMAN β-GLOBIN GENE

Biochemical transformants of mouse L may consti-tute a competent subpopulation in which an unselecta-ble gene can be introduced, along with an unlinked selectable gene, at frequencies higher than in the gen-eral population, Wigler, M., et al., Cell 16: 777-785 (1979). Cotransformation experiments have therefore been carried out in which the Herpes viral tk gene was used as a selectable marker to introduce the human β-globin gene into tk- TCC cells. A cloned Hind III restriction endonuclease fragment of human chromo-somal DNA containing the β-globin gene (plasmid phβ-8) was cleaved with the enzyme Hind III and mixed with Hind III-linearized ptk-1. After TCC tk- cells were exposed to these genes, they were grown for two weeks in HAT selection medium and tk+ transfor-mants were cloned and analyzed by blot hybridization for presence of human β-globin sequences. A 4.3 kb Bgl II restriction fragment containing the intact human β-globin gene is entirely contained within the donor pH −8 plasmid. High molecular weight DNA from the transformants was therefore cleaved with the Bgl/32 II enzyme and analyzed in blot hybridization using the P-labeled 4.3 kb Bgl II fragment as an annealing probe.

In two of the ten TCC transformants examined, human β-globin sequences were detected. One of the transformants contains one to three copies of the 4.3 kb Bgl II fragment; in this cell line, therefore, the globin gene is evidently intact. The other TCC isolate contain-ing the human β-globin gene displays an aberrant high moecular weight annealing fragment, a result suggest-ing that cleavage and integration have occurred within the Bgl II fragment. These data demonstrate that those

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TCC cells that are competent for uptake and expression of the tk gene also integrate another unlinked and unselectable gene at high frequency.

DISCUSSION

The experimental introduction of foreign DNA into early mammalian embryos, and its persistence and augmentation during development, were first reported some six years ago, Jaenisch, R. & Mintz, B., Proc. Natl. Acad. Sci. 71: 1250-1254 (1974). Purified (nonrecombinant) SV 40 viral DNA was microinjected into mouse blastocysts; they gave rise to healthy adults whose tissue DNA contained SV 40 gene sequences. Newer technologies such as described herein should allow a wide range of specific genes to be incorporated into the genome of the embryo for in vivo analyses of control of gene expression during differentiation. With the advent of recombinant DNA, quantities of particular genes in native or specifically modified form can be obtained. In the biological sphere, the malignant stem cells of mouse teratocarcinomas have contributed a novel anenue of intervention. These cells can be grown in culture, selected for specific mutations, and microinjected into blastocysts, where they lose their neoplastic properties and participate in devlopment, Dewey, M., J. et al., Proc. Natl. Acad., Sci. USA, 74: 5564-5568 (1977); Watanabe, T., et al., Proc. Natl. Acad. Sci., 75: 5113-5117 (1978). The cultured TCC cells have therefore been viewed as vehicles for transmitting predetermined genetic changes to mice, Mintz, B., Brookhaven Symp., Bio., 29: 82-85, (1977); Mintz, B., Differentiation 13: 25-27 (1979). Such changes obviously might include genes acquired by uptake of DNA.

DNA-mediated gene transfer into cells of fibroblast lines has been accomplished in culture, Wigler, M., et al., Cell 11: 223-232 (1977); Wigler, M., et al., Cell 14: 725-731 (1978); Willecke, K., et al., Molec. Gen. Genet. 170: 179-185 (1979), Graf, L. H., et al., Somat. Cell Genet., in press (1979); Wigler, M., et al., Proc. Natl. Acad. Sci. USA, 76: 1373-1376 (1979); Wigler, M., et al. Proc. Natl. Acad. Sci., in press (1980); Lewis, W. H. et al., Somat. Cell Genet., in press (1979), and furnished the basis for similar attempts here with teratocarcinoma lines. The TCC-cell route for gene transfer into embryos, as compared with embryo injection of DNA, offers the advantage that transformants, i.e., cell clones in which the specific gene has been retained, can be identified and isolated by selection or screening. In the case of unselectable genes, cotransfer with a selectable one has been found to occur with relatively high frequency, Wigler, M., et al., Cell 16: 777-785 (1979).

In the present study, tk⁻ teratocarcinoma cells have been treated with the cloned thymidine kinase gene of Herpes simplex and a number of HAT-resistant tk+ clones have been obtained with a frequency of about one transformant per μg of DNA. The reason for the markedly lower frequency of TCC transformants than of L-cell transformants, Wigler, M., et al., Cell 14: 725-731 (1978), is obscure since the basis for transformation competence in eucaryotic cells remains unknown. The donor origin of the tk+ phenotype in the TCC transformants was demonstrated by the HSV-type electrophoretic mobility of their tk enzyme, and also by neutralization of the tk activity by specific antiserum raised against HSV-1 tk (Table I). Furthermore, blot hybridization tests indicated that at least one intact copy of the viral tk gene was present and integrated into other DNA in the transformed cells. These data support

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the conclusion that the tk activity in the transformed clones is indeed attributable to presence and expression of the viral gene.

A requirement for experiments involving the introduction of genes is that they remain stable in vivo, even in the absence of selective pressure, during many cell generations. Stability of the tk⁺ transformed phenotype was in fact not only in culture (Table II), but also in tumors arising after subcutaneous inoculation of the stem cells into mice. These tumors exhibited various types of tissue differentiation, similar to the range observed in the untransformed parent TCC line. Hybridization experiments comparing each tumor with its transformed cell line of origin indicated that the donor tk gene was maintained without significant loss or rearrangement in seven of nine tumors examined.

Many genes of interest in a developmental context are not selectable. An example is the globin gene. As in related experiments with L-cells, Wigler, M., et al., Cell 16: 777-785 (1979), a fragment of human genomic DNA containing an intact β-globin gene was administered to TCC tk⁻ cells along with the unlinked HSV tk gene. This proved to be an effective method to obtain TCC tk+ clones in which, from hybridization evidence, the human β-globin gene was present.

The experiments described herein therefore demonstrate that cultured TCC stem cells can accept exogenous genes and that such genes can be stably retained as well as expressed during in vivo differentiation in tumors. On this basis, experiments with a euploid TCC cell line can proceed, for the purpose of creating in vivo markers appropriate for analyses of gene regulation during embryogenesis.

MATERIALS AND METHODS

Cell Cultures

Ltk aprt, a derivative of Ltk clone D, Kit, S. et al., Esp. Cell Res. 31:291-312 (1963), was maintained in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum (Flow Laboratories, Rockville, MD.) and 50 μg/ml of diaminopurine (DAP). Prior to transformation, cells were washed and grown for three generations in the absence of DAP. A Chinese hamster cell line containing an altered dihydrofolate reductase (rendering it resistant to methoxtrexate) A29 MtxRIII, Flintoff, W. F., et al, Somatic Cell Genetics 2:245-261 (1976), was propogated in DME supplemented with 3 × non-essential amino acids, 10% calf serum and 1 μg/ml amethopterin. For the amplification experiments, the medium was additionally supplemented with 20 μg/ml of methotrexate.

Murine Ltk⁻aprt⁻cells are adenine phosphoribosyltransferase-negative derivaties of Ltk⁻clone D cells. Cells were maintained in growth medium and prepared for transformation as described, Wigler, M., et al, PNAS 76:1373-1376 (1979).

HEp-2(human), HeLa(human), CHO (Chinese hamster ovary), and Ltk⁻cells were gown in growth medium. LH2b, a derivative of Ltk⁻transformed with herpes simples virus tk DNA, was maintained in growth medium containing hypoxanthine at 15 μg/ml, aminopterin at 0.2 μg/ml, and thymidine at 5.0 μg/ml (HAT), Wigler, M., et al ., Cell 1:223-232 (1977). All culture dishes were Nunclon (Vanguard International, Neptune, N.J.) plastic.

The feeder-independent mouse teratocarcinoma cell culture line 6050P, Watanabe, T., et al., PNAS

75:5113–5117 (1978), obtained from a tumor of the OTT 6050 transplant line, was used as the wild-type, or tk+, parent and is here designated TCC wt. This line is of the X/O sex chromosome type and has a modal number of 39 chromosomes with characteristics described in Watanabe, T., et al., (1978). The cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. After 3 hr of exposure to 3 μg/ml of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine, the cells were allowed to recover for two days and were then transferred to medium with 80 μg/ml of BrdUrd. A series of resistant clones were isolated; one supplied the clonal line (TCC tk−) used in the present transformation experiments. This line had a revision frequency to wild-type of less than 10−8. The cells were maintained in medium with 30 μg/ml of BrdUrd and, prior to transformation, were washed and grown for three generations in the absence of the drug. Transformation efficiency was compared with that of a tk-deficient line, Kit, S., et al., Exp. Cell. Res. 31:297–312 (1963) of mouse L-cells (L tk−).

Extraction and Restriction Endonuclease Cleavage of Genomic DNA

High molecular weight DNA was obtained from cultured cells (CHO, LH2b, and HeLa) or from frozen rabbit livers as previously described. Wigler, M., et al., Cell 14:725–731 (1978). High molecular weight salmon sperm DNA was obtained from Worthington. Restriction endonuclease cleavage (Bam I, HindIII, Kpn I, and Xba I) was performed in a buffer containing 50 mM NaCl, 10 mM Tris.HCL, 5 mM MgCl$_2$, 7 mM mercaptoethanol, and bovine serum albumin at 100 μg/ml (pH 7.9). The enzyme-to-DNA ratio was at least two units/μg of DNA, and reaction mixtures were incubated at 37° C. for at least 2 hrs (one unit is the amount of enzyme that digests 1 μg of DNA in 1 hr). To monitor the completeness of digestion, 1 μl of nick-translated adenovirus-2 [^{32}P]DNA was incubated with 5 μl of reaction volume for at least 2 hr, cleavage products were separated by electrophoresis in 1% agarose gels, and digestion was monitored by exposing the dried gel to Cronex 2DC x-ray film.

Intact herpes simplex virus (HSV) DNA was isolated from CV-1-infected cells as previously described. Pellicer, A., et al., Cell 14:133–141 (1978). DNA was digested to comletion with Kpn 1 (New England Biolabs) in a buffer containing 6 mM Tris (pH 7.9), 6mM MgCl$_2$, 6 mM 2-mercaptoethanol, 6 mM NaCl and 200 μg/ml bovine serum albumin. The restricted DNA was fractionated by electrophoresis through 0.5% agarose gels (17×20×0.5 cm) for 24 hr at 70 V, and the 5.1 kb tk-containing fragment was extracted from the gel as described by Maxam, A. M. and Gilbert, W. PNAS 74.560–564 (1977) and Wigler, M., et al., Cell 14:725–731 (1978)

ΦX174 am3 RFI DNA was purchased ffrom Bethesda Research Laboratories. Plasmid pBR322 DNA was grown in E. coli HB 101 and purified according to the method of Clewell, D. B., J. Bacteriol. 110·667–676 (1972). The cloned rabbit β major globin gene in the λ Charon4A derivative (RβG-1) was identified and isolated as previously described. Maniatis, T., et al., Cell 15:687–701 (1978).

In the amplification experiments, the size of the high molecular weight DNA was determined by electrophoresis in 0.3% agarose gels using herpes simplex virus DNA and its Xba I fragments as markers. Only DNA whose average size was larger than 75 kb was found to possess transforming activity in the amplification experiments. In these experiments, plasmid DNAs were isolated from chloramphenicol amplified cultures by isopycnic centrifugation in CsCl gradients containing 300 μg/ml ethidium bromide.

TRANSFORMATION AND SELECTION

The transformation protocol was as described in Graham, F. L. and Van der Eb, A. J., Virology. 52.456–457 (1973) with the following modifications. One day prior to transformation, cells were seeded at 0.7×10^6 cells per dish. The medium was changed 4 hr prior to transformation. Sterile, ethanol-precipitated high molecular weight or restriction endonuclease-cleaved eucaryotic DNA dissolved in 1 mM Tris (pH 7.9)/0.1 mM EDTA was used to prepare DNA/CaCl$_2$ which contains DNA at 40 μg/ml and 250 mM CaCl$_2$ (Mallinkrodt). Twice-concentrated Hepes-buffered saline (2× HBS) was prepared; it contains 280 mM NaCl, 50 mM Hepes, and 1.5 mM sodium phosphate, pH adjusted to 7.10±0.05 DNA/CaCl$_2$ solution was added dropwise to an equal volume of sterile 2× HBS. A 1-ml sterile plastic pipette with a cotton plug was inserted into the mixing tube containing 2× HBS, and bubbles were introduced by blowing while the DNA was being added. The calcium phosphate/DNA precipitate was allowed to form without agitation for 30–45 min at room temperature. The precipitate was then mixed by gentle pipetting with a plastic pipette, and 1 ml of precipitate was added per plate, directly to the 10 ml of growth medium that covered the recipient cells. After 4-hr incubation at 37° C., the medium was replaced and the cells were allowed to incubate for an additional 20 hr. At that time, selective pressure was applied. For tk+ selection, medium was changed to growth medium containing HAT. For aprt+ selection, cells were trypsinized and replated at lower density (about 0.5×10^6 cells per 10-cm dish) in medium containing 0.05 mM azaserine and 0.1 mM adenine. For both tk+ and aprt+ selection, selective media were changed the next day, 2 days after that, and subsequently every 3 days for 2–3 weeks while transformant clones arose. Colonies were picked by using cloning cylinders and the remainder of the colonies were scored after formaldehyde fixation and staining with Giemsa. For characterization, clones were grown into mass culture under continued selective pressure. A record was kept of the apparent number of cell doublings for each clone isolated.

Methotrexate-resistant transformants of Ltk− aprt− cells were obtained following transformation with 20 μg of high molecular weight DNA from A29 Mtx^{RIII} cells and selection in DME containing 10% calf serum and 0.2 μg/ml amethopterin.

For tk+ selection, cells were grown in HAT medium, for resistance to methotrexate, cells were selected in medium supplemented with 0.1 μg/ml of methotrexate. Colonies were cloned from individual dishes to assure that each transformant arose from an independent event. Ligates between A29 and linearized pBR322 DNA were prepared by incubating a 1:1 ration (w/w) of Sal I-cleaved DNAs with T$_4$ ligase (Bethesda Research Laboratories) under the conditions recommended by the supplier. A calcium phosphate precipitate was prepared using 2 μg ligate and 18 μg carrier/ml, and added to recipient cells (the amount of ligate was limited because of the observation that plasmid inhibits transformation). The DNA was allowed to

remain in contact with the cells for 4–12 hr and the medium was then aspirated and replaced with fresh DME. Selective pressure was applied 24 hr f...owing exposure to DNA. After 2-3 weeks, colonies were isolated using cloning cylinders.

In the mouse teratocarcinoma cell experiments, transformation was performed as described previously except that the TCC tk⁻ cells were seeded at 3×10⁵ cells/plate one day prior to transformation. To each plate of attached cells was added a calcium phosphate/DNA precipitate prepared with 4 μg of the recombinant plasmid, Ptk-1, digested with Bam H1, in the presence of 20 μg of high molecular weight DNA obtained from L tk⁻ aprt⁻ cells.

In addition, some cells were treated in suspension, Willecke, K. et al., Molec. Gen. Genet. 170:179-185 (1979). 7×10⁶ freshly trypsinized TCC tk⁻ cells were mixed with a calcium phosphate/DNA precipitate prepared with 10 μg of DNA from the BAM H1-claved plasmid Ptk-1 and 150 μg of high molecular weight DNA from salmon sperm. Following centrifugation, resuspension, and shaking, as described in Willecke, K. et al. (1979), the cells were again plated in growth medium. After three days, the medium was replaced with HAT medium and colonies of transformants were isolated after two weeks.

Cotransformation experiments were performed with 4 μg of Bam H1-digested Ptk-1 DNA along with 4 μg of Hind III-cleaved plasmid pHβ-8 containing the chromosomal adult human β-globin gene, Lawn, R. M., et al., Cell 15:1157-1174 (1978). Tk⁺ transformants were selected in growth medium containing 0.1 mM hypoxanthine/0.4 μM aminopterin/16 μM thymidine(HAT). Colonies were picked with cloning cylinders and were grown into mass cultures.

COTRANSFORMATION OF DEFINED DNA SEQUENCES AND THE HSV TK GENE

Ltk⁻ aprt⁻ mouse cells were transformed with either 1–10 μg of ΦX174, 1 μg of pBR322 or 1 μg of RβG-1 DNA in the presence of 1 ng of HSV-1 tk gene and 10-20 μg of salmon sperm carrier DNA, as previously described. Wigler, M. et al., PNAS 76:1373–1376 (1979). Tk⁺ transformants were selected in DME containing hypoxanthine, aminopterin and thymidine (HAT) and 10% calf serum. Isolated colonies were picked using cloning cylinders and grown into mass cultures.

ENZYME ASSAYS

Extracts were prepared by resuspending washed cell pellets (approximately 10⁷ cells) in 0.1 ml of 0.02 M potassium phosphate, pH 7, containing 0.5% Triton X-100. The supernatant (cytoplasm) obtained after 25 min of 700×g centrifugation was used for the quantitation of enzymatic activity and for electrophoresis. aprt and protein were assayed as previously described. Chasin L. A., Cell 2:37–41 (1974). Inclusion of 3 mM thymidine triphosphate, an inhibitor of 5'-nucleotidase, Murray, A. W. and Friedrichs, B., Biochem., J. 111:83-89 (1969), in the reaction mixture did not increase AMP recovery, indicating that the nucleotidase was not interfering with the measurement of aprt activity. Isoelectric focusing of aprt was carried out essentially as described for hypoxanthine phosphoribosyltransferase, Chasin, L. A. and Urlaub, G. Somat. Cell Genet. 2:453-467 (1976), with the following exceptions: The polyacrylamide gel contained an Ampholine (LKB) mixture of 0.8% pH 2.5-4,

0.8% pH 4–6, and 0.4% pH 5-7. For assaying enzymatic activity, [2-³H] adenine [0.04 mM, 1 Ci/mmol, New England Nuclear (1 Ci=3.7×10¹⁰ becquerels)] was substituted for hypoxanthine.

ASSAYS OF THYMIDINE KINASE ACTIVITY

For specific activity measurements, cells from monolayer cultures were scraped into phosphate buffered saline and washed. The cell pellet was suspended in 5 volumes of extraction buffer (0.01 M Tris.HCl, pH 7.5, 0.01 M KCl, 1mMMgCl₂, 1mM 2-mercaptoethanol, and 50 μM thymidine). The cell suspension was frozen and thawed three times and the KCl concentration was then adjusted to 0.15 M. After sonication, the cytoplasmic extract was obtained by centrifugation at 30,000×g for 30 min, and the supernatant was used for tk assays as described in Wigler, M. et al. Cell 16:777-785 (1979). Cytoplasmic extracts from tumors were obtained after disruption of the cells in a Potter-Elvejehm homogenizer. They were then treated as described above for cultured cells. One unit of thymidine kinase is defined as the amount of enzyme which converts one nanomole of thymidine into thymidine monophosphate per minute.

In enzyme neutralization studies, anti-HSV-1 tk antiserum or preimmune serum was mixed with an equal volume of cytoplasmic extract, and ATP and magnesium were added to 6.7 mM. The enzyme-antibody mixture was incubated for 30 min at room temperature, centrifuged at 2,000×g for 10 min, and the supernatant was assayed for tk activity.

In an additional biochemical assay, 30,000×g supernatants of homogenates from cell cultures and from solid tumors were electrophoresed on 5% polyacrylamide gels which were then cut into 1.6 mm slices and assayed for tk activity as described. Lee, L. S. and Cheng, Y. C., J. Biol. Chem., 251:2600-2604 (1976).

RNA Isolation

Total RNA was isolated from logarithmic-phase cultures of transformed L cells by successive extractions with phenol at pH 5.1, phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol), and chloroform/isoamyl alcohol (24:1, vol/vol). After ethanol precipitation, the RNA was digested with DNase, Maxwell, I. H., et al., Nucleic Acids Res. 4:241-246 (1977) and precipitated with ethanol. Nuclear and cytoplasmic fractions were isolated as described in Wigler, M. et al., PNAS 76:1373-1376 (1979) and RNAs were extracted as described above. Cytoplasmic polyadenylylated RNA was isolated by oligo(dT)-cellulose chromatography. Axel, R. et al., Cell 7:247-254 (1976).

CDNA SYNTHESIS

Rabbit and mouse cDNAs were prepared by using avian myeloblastosis virus reverse transcriptase (RNA-dependent DNA polymerase) as described in Myers, J. C. and Spiegelman, S., PNAS 75:5329-5333 (1978).

ISOLATION OF TRANSFORMED CELL DNA

Cells were harvested by scraping into PBS and centrifuging at 1000×g for 10 min. The pellet was resuspended in 40 vol of TNE [10 mM Tris-HCl (ph 8.0), 150 mM NaCl, 10 mM EDTA], and SDS and proteinase K were added to 0.2% and 100 μg/ml, respectively. The lysate was incubated at 37° C. for 5-10 hr and then extracted sequentially with buffer-saturated phenol and CHCl₃. High molecular weight DNA isolated by mixing the aqueous phase with 2 vol of cold ethanol and

immediately removing the precipitate that formed. The DNA was washed with 70% ethanol and dissolved in 1 mM Tris, 0.1 EDTA.

Nuclei and cytoplasm from clones ΦX4 and ΦX5 were prepared as described by Ringold, G. M., et al. Cell 10:19-26 (1977). The nuclear fraction was further fractionated into high and low molecular weight DNA as described by Hirt, B., J. Mol. Biol. 26:365-369 (1967).

DNA FILTER HYBRIDIZATIONS

Cellular DNA was digested with restriction endonucleases, electrophoresed on agarose slab gels, transferred to nitrocellulose filter sheets, and hybridized with 32P-labeled DNA probes as described by Wigler, M. et al., PNAS 76:1373-1376 (1979).

DNA from transformed cells was digested with various restriction endonucleases using the conditions specified by the supplier New England Biolabs or Bethesda Research Laboratories). Digestions were performed at an enzyme to DNA ratio of 1.5 U/μg for 2 hr at 37° C. Reactions were terminated by the addition of EDTA, and the product was electrophoresed on horizontal agarose slab gels in 36 mM Tris, 30 mM NaH$_2$PO$_4$, 1 mM EDTA (pH 7.7). DNA fragments were transferred to nitrocellulose sheets, hybridized and washed as previously described. Weinstock, R., et al., PNAS 75:1299-1303 (1978) with two modifications. Two nitrocellulose filters were used during transfer. Jeffreys, A. J. and Flavell, R. A., Cell 12:1097-1108 (1977). The lower filter was discarded, and following hybridization the filter was washed 4 times for 20 min in 2×SSC, 25 mM sodium phosphate, 1.5 mM Na$_4$P$_2$O$_7$, 0.05% SDS at 65° C. and then successively in 1:1 and 1:5 dilutions of this buffer. Jeffreys, A. J. and Flavell, R. A., Cell 12:429-439 (1977).

In the amplification experiments the probes were either 32P-nick translated pBR322 or pdhfr-21, a cDNA copy of mouse dhfr mRNA. Chang, A.C.Y., et al., Nature 275:617-624 (1978).

SOLUTION HYBRIDIZATIONS

32P-Labeled globin cDNAs (specific activities of 2-9×10^8 cpm/μg) were hybridized with excess RNA in 0.4 M NaCl/25 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.5/5 mM EDTA at 75° C. Incubation times did not exceed 70 hr. Rots were calculated as moles of RNA nucleotides per liter times time in seconds. The fraction of cDNA rendered resistant to the single-strand nuclease S1- in hybridization was determined as described. Axel, R. et al., Cell 7:247-254 (1976).

RNA FILTER HYBRIDIZATION

RNA was electrophoresed through 1% agarose slab gels (17×20×0.4 cm) containing 5 mM methylmercury hydroxide as described by Bailey, J. and Davidson, N., Anal. Biochem. 70:75-85 (1976). The concentration of RNA in each slot was 0.5 μg/μl. Electrophoresis was at 110 V for 12 hr at room temperature.

RNA was transferred from the gel to diazotized cellulose paper as described by Alwine, J. C., et al., PNAS 74:5350-5354 (1979) by using pH 4.0 citrate transfer buffer. After transfer, the RNA filter was incubated for 1 hr with transfer buffer containing carrier RNA at 500 μg/ml. The RNA on the filters was hybridized with cloned DNA probe at 50 ng/ml labeled by 32P-nick translation, Weinstock, R., et al., PNAS 75.1299-1303 (1978) to specific activities of 2-8×10^8 cpm/μg. Reaction volumes were 25 μl/cm^2 of filter. Hybridization

was in 4× standard saline citrate (0.15 M NaCl/0.015 M sodium citrate)/50% formamide at 57° C. for 36-48 hr.

After hybridization, filters were soaked in two changes of 2× standard saline citrate/25 mM sodium phosphate/1.5 mM sodium pyrophosphate/0.1% sodium dodecyl sulfate/5 mM EDTA at 37° C. for 30 min with shaking to remove formamide. Successive washes were at 68° C. with 1× and 0.1× standard saline citrate containing 5 mM EDTA and 0.1% sodium dodecyl sulfate for 30 min each.

BERK SHARP ANALYSIS OF RABBIT β-GLOBIN RNA IN TRANSFORMED MOUSE L CELLS

The hybridizations were carried out in 80% (vol/vol) formamide (Eastman)/0.4 M Pipes, pH 6.5/0.1 mM EDTA/0.4 M NaCl, Casey, J. and Davidson, N., Nucleic Acid Res., 4:1539-1552 (1977); Berk, A. J. and Sharp, P. A., Cell 12:721-732 (1977)for 18 hr at 51° C. for the 1.8 kbp Hha I fragment and 49° C. for the Pst I fragment. The hybrids were treated with S1 nuclease and analyzed essentially by the procedure described by Berk, A. J. and Sharp, P. A. (1977).

Although the instant disclosure sets forth all essential information in connection with the invention, the numerous publications cited herein may be of assistance in understanding the background of the invention and the state of the art. Accordingly, all of the publications cited are hereby incorporated by reference into the present disclosure.

What is claimed is:

1. A process for inserting foreign DNA I into a suitable eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I and with unlinked foreign DNA II which codes for a selectable phenotype not expressed by said ducaryotic cell, said cotransformation being carried out under suitable conditions permitting survival or identification of eucaryotic cells which have acquired said selectable phenotype, said foreign DNA I being incorporated into the chromosomal DNA of said eucaryotic cell.

2. A process in accordance with claim 1 wherein said foreign DNA I codes for proteinaceous material which is not associated with a selectable phenotype.

3. A process in accordance with claim 2 wherein said foreign DNA I codes for interferon protein.

4. A process in accordance with claim 2 wherein said foreign DNA I codes for insulin.

5. A process in accordance with claim 2 wherein said foreign DNA I codes for growth hormone.

6. A process in accordance with claim 2 wherein said foreign DNA I codes for a clotting factor.

7. A process in accordance with claim 2 wherein said foreign DNA I codes for a viral antigen or an antibody.

8. A process in accordance with claim 2 wherein said foreign DNA I codes for an enzyme.

9. A process in accordance with claim 1 wherein said foreign DNA I is substantially purified.

10. A process in accordance with claim 1 wherein said foreign DNA I has been obtained from restriction endonuclease cleavage of eucaryotic chromosomal DNA.

11. A process in accordance with claim 1 wherein said foreign DNA I and DNA II have been treated with calcium phosphate.

12. A process in accordance with claim 1 wherein said eucaryotic cell is a mammalian cell.

13. A process in accordance with claim 12 wherein said mammalian cell is an erythroblast.

14. A process in accordance with claim 12 wherein said mammalian cell is a fibroblast.

15. A process in accordance with claim 1 wherein said foreign DNA I is present in an amount relative to said DNA II which codes for a selectable phenotype in the range from about 1:1 to about 100,000:1.

16. A process in accordance with claim 1 wherein said DNA II which codes for a selectable phenotype comprises the gene for thymidine kinase from herpes simplex virus.

17. A process in accordance with claim 1 wherein said DNA II which codes for proteinaceous material which is associated with a selectable phenotype comprises the gene for adenine phosphoribosyltransferase.

18. A process in accordance with claim 1 wherein said DNA II which codes for a selectable phenotype comprises a gene associated with drug resistance.

19. A process in accordance with claim 18 wherein said gene associated with drug resistance is the gene coding for a mutant dihydrofolate reductase which renders cells resistant to methotrexate.

20. A eucaryotic cell into which foreign DNA I has been inserted in accordance with the process of claim 1.

21. A mammalian cell into which foreign DNA I has been inserted in accordance with the process of claim 1.

22. A process for producing a foreign proteinaceous material which comprises cotransforming a eucaryotic cell in accordance with the process of claim 1, culturing or cloning said cotransformed eucaryotic cell under suitable conditions to yield a multiplicity of eucaryotic cells producing said foreign proteninaceous material and recovering said proteinaceous material from said eucaryotic cells.

23. A process in accordance with claim 22 wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

24. A process in accordance with claim 22 wherein said eucaryotic cell is a mammalian cell.

25. A method of detecting eucaryotic cells which have been transformed with foreign DNA I which is not associated with a selectable phenotype which comprises cotransforming said eucaryotic cell with said DNA I and with DNA II which is associated with a selectable phenotype in accordance with the process of claim 1, and screening for eucaryotic cells so cotransformed.

26. A process for inserting foreign DNA I into a eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I and with unlinked foreign DNA II which codes for a selectable phenotype not expressed by said eucaryotic cell, said cotransformation being carried out in a suitable medium and in the presence of conditions permitting identification and recovery of eucaryotic cells which have acquired said selectable phenotype.

27. A process for cotransforming a suitable eucaryotic cell which comprises transforming under suitable conditions said eucaryotic cell with foreign DNA I and with foreign DNA II, said DNA I and DNA II being unlinked and said DNA II coding for a selectable phenotype not expressed by said eucaryotic cell prior to cotransformation.

28. A process for inserting purified foreign DNA I coding for proteinaceous material which is not associated with a selectable phenotype into a suitable eucaryotic cell which comprises cotransforming said eucaryotic cell with said foreign DNA I and with unlinked foreign DNA II coding for proteinaceous material which is associated with a selectable phenotype, said cotransformation being carried out under suitable conditions permitting survival or identification of eucaryotic cells which have acquired said selectable phenotype, said foreign DNA I being incorporated into the chromosomal DNA of said eucaryotic cell.

29. A process in accordance with claim 28 wherein said proteinaceous material which is not associated with a selectable phenotype comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

30. A eucaryotic cell into which foreign DNA I has been inserted in accordance with the process of claim 28.

31. A process for inserting a multiplicity of foreign DNA I molecules corresponding to multiple copies of a gene coding for a proteinaceous material into a suitable eucaryotic cell which comprises cotransforming said eucaryotic cell with said multiplicity of foreign DNA I molecules and with a multiplicity of unlinked foreign DNA II molecules coding for a selectable phenotype not expressed by said eucaryotic cell, said cotransformation being carried out under suitable conditions permitting survival or identification of eucaryotic cells which have acquired said multiplicity of genes coding for said selectable phenotype.

32. A process in accordance with claim 31 wherein said foreign DNA I codes for proteinaceous material which is not associated with a selectable phenotype.

33. A process in accordance with claim 32 wherein said foreign DNA I codes for interferon protein.

34. A process in accordance with claim 32 wherein said foreign DNA I codes for insulin.

35. A process in accordance with claim 32 wherein said foreign DNA I codes for growth hormone.

36. A process in accordance with claim 32 wherein said foreign DNA I codes for a clotting factor.

37. A process in accordance with claim 32 wherein said foreign DNA I codes for a viral antigen or an antibody.

38. A process in accordance with claim 32 wherein said foreign DNA I codes for an enzyme.

39. A process in accordance with claim 31 wherein said foreign DNA I is substantially purified.

40. A process in accordance with claim 31 wherein said foreign DNA I has been obtained from restriction endonuclease cleavage of eucaryotic chromosomal DNA.

41. A process in accordance with claim 31 wherein said foreign DNA I and DNA II have been treated with calcium phosphate.

42. A process in accordance with claim 31 wherein said eucaryotic cell is a mammalian cell.

43. A process in accordance with claim 42 wherein said mammalian cell is an erythroblast.

44. A process in accordance with claim 42 wherein said mammalian cell is a fibroblast.

45. A process in accordance with claim 31 wherein said foreign DNA I is present in an amount relative to said DNA II which codes for proteinaceous material associated with a selectable phenotype in the range from about 1:1 to about 100,000:1.

46. A process in accordance with claim 31 wherein said foreign DNA II which does for proteinaceous

material which is associated with a selectable phenotype comprises a gene associated with drug resistance.

47. A process in accordance with claim 46 wherein said gene associated with drug resistance is a gene coding for a mutant dihydrofolate reductase which renders cells resistant to methotrexate.

48. A process in accordance with claim 31 wherein said foreign DNA I is incorporated into the chromosomal DNA of said eucaryotic cell.

49. A eucaryotic cell into which foreign DNA I has been inserted in accordance with the process of claim 31.

50. A mammalian cell into which foreign DNA I has been inserted in accordance with the process of claim 31

51. A process for producing a foregin proteinaceous material which comprises cotransforming a eucaryotic cell in accordance with the process of claim 31, maintaining said cotransformed eucaryotic cell under suitable conditions to produce said foreign proteinaceous material, and recovering said proteinaceous material so produced.

52. A process in accordance with claim 51 wherein said proteinaceous material comprises interferon protein, insulin, growth hormone, clotting factor, viral antigen or antibody.

53. A process in accordance with claim 51 wherein said eucaryotic cell is mammalian cell.

54. A process for generating a multiplicity of foregin DNA I molecules corresponding to multiple copies of a gene in a eucaryotic cell which comprises tranforming said eucaryotic cell with a molecule which is formed by linking one of said foreign DNA I molecules to a DNA II molecule corresponding to an amplifiable gene for a dominant selectable phenotype not expressed by said eucaryotic cell, and culturing the transformed eucaryotic cells in the presence of successively elevated concentrations of an agent permitting survival or identification of eucaryotic cells which have acquired multiple copies of said amplifiable gene, said transformation and culturing being carried out under suitable conditions.

55. A process in accordance with claim 54 wherein said foreign DNA I codes for proteinaceous material which is not associated with a selectable phenotype.

56. A process in accordance with claim 55 wherein said foreign DNA I codes for interferon protein.

57. A process in accordance with claim 55 wherein said foreign DNA I codes for insulin.

58. A process in accordance with claim 55 wherein said foreign DNA I codes for growth hormone.

59. A process in accordance with claim 55 wherein said foreign DNA I codes for a clotting factor.

60. A process in accordance with claim 55 wherein said foreign DNA I codes for a viral antigen or antibody.

61. A process in accordance with claim 55 wherein said foregin DNA I codes for an enzyme.

62. A process in accordance with claim 54 wherein said foreign DNA I is substantially purified.

63. A process in accordance with claim 54 wherein said foreign DNA I has been obtained from restriction endonuclease cleavage of eucaryotic chromosomal DNA.

64. A process in accordance with claim 54 wherein said foreign DNA I and DNA II have been treated with calcium phosphate.

65. A process in accordance with claim 54 wherein said eucaryotic cell is mammalian cell.

66. A process in accordance with claim 65 wherein said mammalian cell is an erythroblast.

67. A process in accordance with claim 65 wherein said mammalian cell is a fibroblast.

68. A process in accordance with claim 54 wherein said foreign DNA I is present in an amount relative to said DNA II which codes for proteinaceous material associated with a selectable phenotype in the range from about 1:1 to about 100,000:1.

69. A process in accordance with claim 54 wherein said DNA II which codes for proteinaceous material which is associated with a selectable phenotype comprises a gene associated with resistance to a drug or chemical antagonist.

70. A process in accordance with claim 69 wherein said gene associated with resistance to a drug or chemical anatgonist is a gene coding for a mutant dihydrofolate reductase which renders cells resistant to methotrexate.

71. A process in accordance with claim 54 wherein said foreign DNA I is incorporated into the chromosomal DNA of said eucaryotic cell.

72. A eucaryotic cell into which foreign DNA I has been inserted in accordance with the process of claim 54.

73. A mammalian cell into which foreign DNA I has been inserted in accordance with the process of claim 54.

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United States Patent [19]

Morrison et al.

[11] **Patent Number:** 5,807,715

[45] **Date of Patent:** Sep. 15, 1998

[54] **METHODS AND TRANSFORMED MAMMALIAN LYMPHOCYTE CELLS FOR PRODUCING FUNCTIONAL ANTIGEN-BINDING PROTEIN INCLUDING CHIMERIC IMMUNOGLOBULIN**

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[21] Appl. No.: **266,154**

[22] Filed: **Jun. 27, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 893,610, Jun. 3, 1992, abandoned, which is a continuation of Ser. No. 675,106, Mar. 25, 1991, abandoned, which is a continuation of Ser. No. 441,189, Nov. 22, 1989, abandoned, which is a continuation of Ser. No. 90,669, Aug. 28, 1987, abandoned, which is a continuation-in-part of Ser. No. 644,473, Aug. 27, 1984, abandoned.

[51] Int. Cl.^{6} C12N 15/00; C12N 15/13; C07K 16/00

[52] U.S. Cl. 435/69.6; 435/172.3; 435/326; 530/387.1; 530/387.3; 536/23.53

[58] Field of Search 435/69.6, 246.27, 435/320.1, 172.3, 326; 530/387.3, 387.1; 935/15; 536/23.53

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[57] ABSTRACT

Methods for producing functional immunoglobulin are provided. The methods involve transfecting and expressing exogenous DNA coding for the heavy and light chains of immunoglobulin. In some embodiments, chimeric immunoglobulins are provided having variable regions from one species and constant regions from another species by linking DNA sequences encoding for the variable regions of the light and heavy chains from one species to the constant regions of the light and heavy chains respectively from a different species. Introduction of the resulting genes into mammalian host cells under conditions for expression provides for production of chimeric immunoglobulins having the specificity of the variable region derived from a first species and the physiological functions of the constant region from a different species.

**62 Claims, 2 Drawing Sheets**
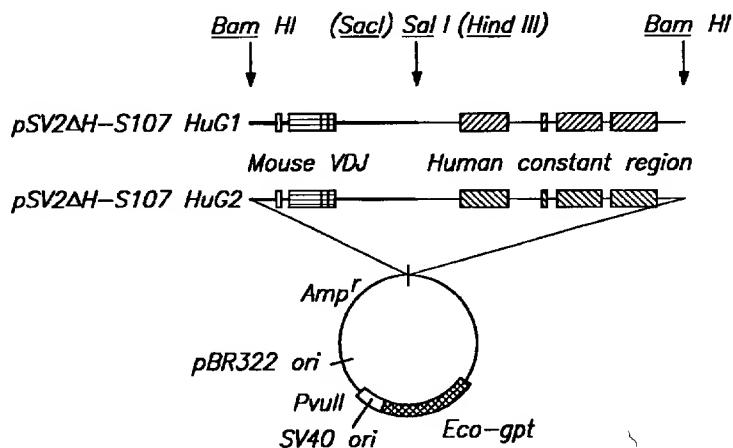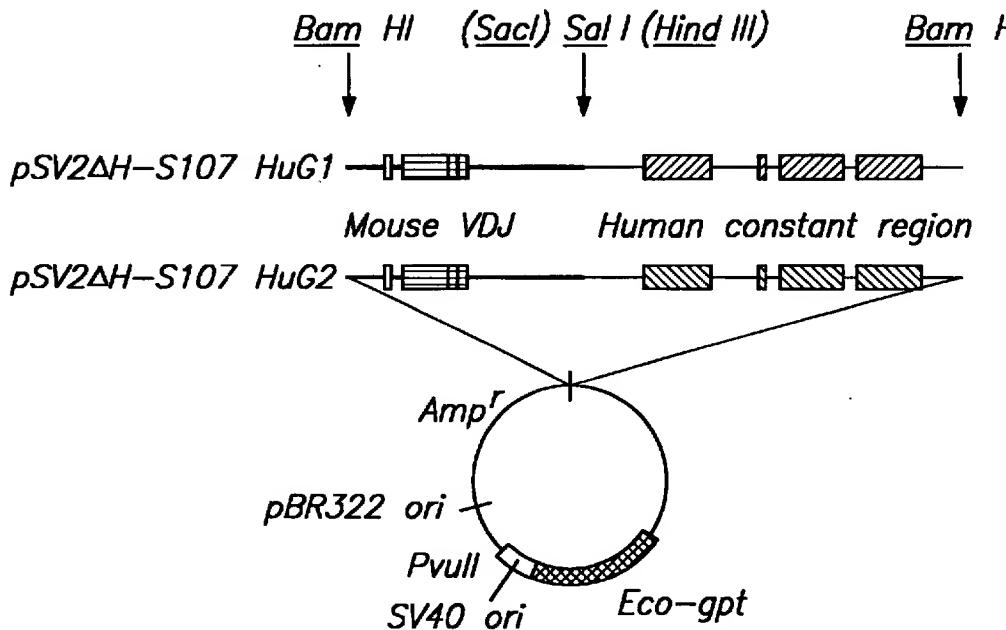
FIG. 1A
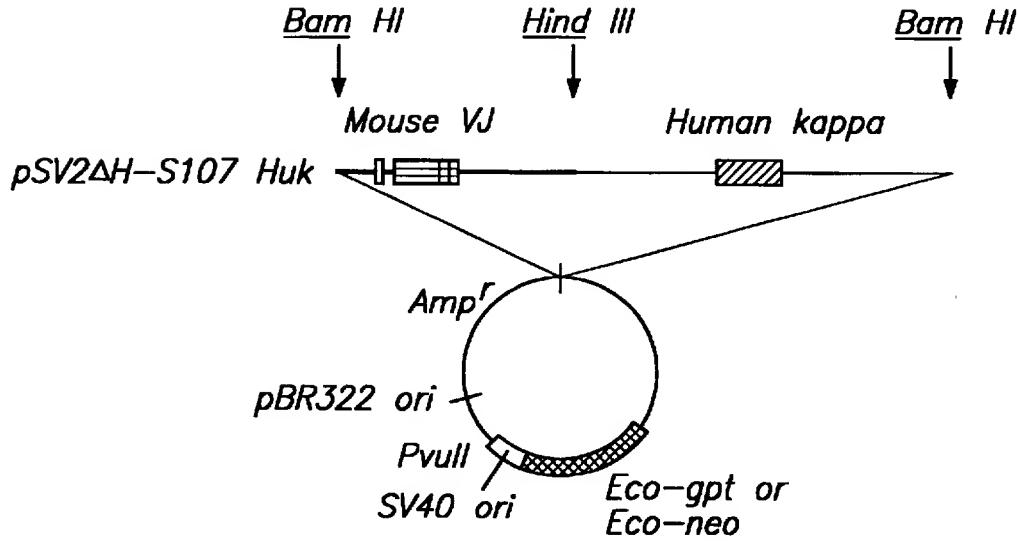
FIG. 1B
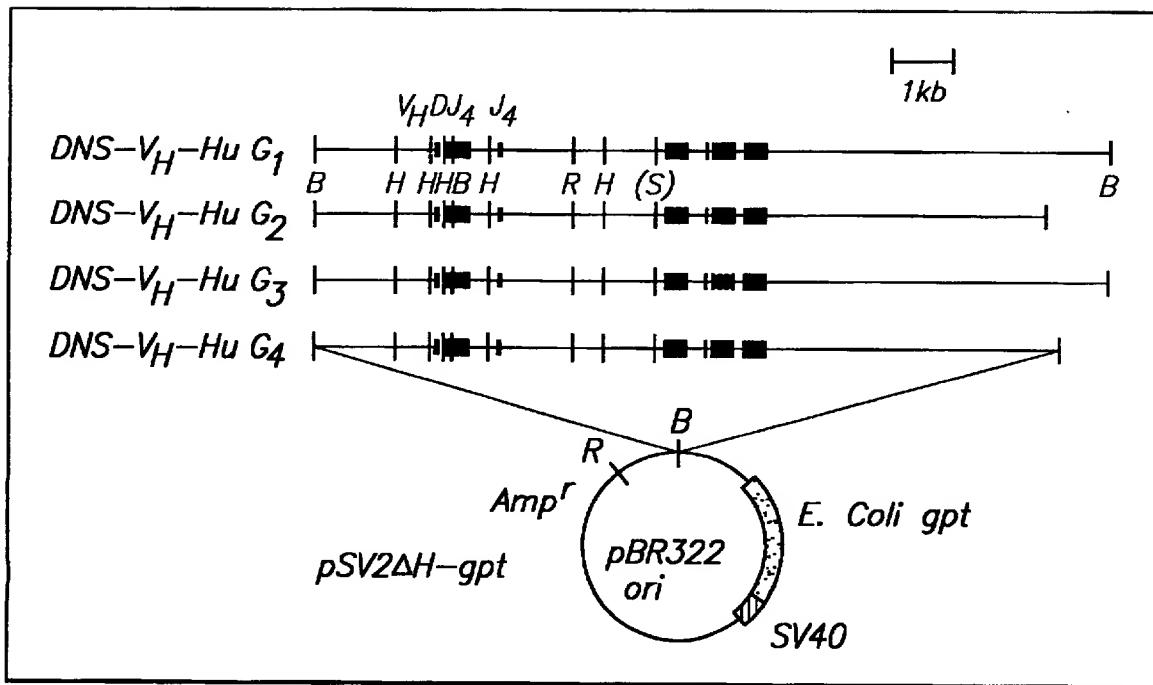
FIG. 2

METHODS AND TRANSFORMED MAMMALIAN LYMPHOCYTE CELLS FOR PRODUCING FUNCTIONAL ANTIGEN-BINDING PROTEIN INCLUDING CHIMERIC IMMUNOGLOBULIN

This is a continuation of application Ser. No. 07/893,610, filed Jun. 3, 1992, now abandoned, which is a continuation of application Ser. No. 07/675,106, filed Mar. 25, 1991, now abandoned, which is a continuation of application Ser. No. 07/441,189, filed Nov. 22, 1989, now abandoned, which is a continuation of application Ser. No. 07/090,669, filed Aug. 28, 1987, now abandoned, which is a continuation-in-part of application Ser. No. 06/644,473, filed Aug. 27, 1984 now abandoned.

BACKGROUND OF THE INVENTION

A. Field of the Invention

Naturally occurring receptors, such as immunoglobulins, enzymes, and membrane proteins have seen an extraordinary expansion in commercial applications over the last decade. With the advent of monoclonal antibodies, the usefulness of immunoglobulins has been greatly expanded and in many situations has greatly extended prior uses employing polyclonal antibodies. However, in many applications, the use of monoclonal antibodies is severely restricted where the monoclonal antibodies are to be used in a physiological (in vivo) environment. Since, for the most part, monoclonal antibodies are produced in rodents, e.g., mice, the monoclonal antibodies are immunogenic to other species.

While the constant regions of immunoglobulins are not involved in ligand binding, the constant regions do have a number of specific functions, such as complement binding, immunogenicity, cell receptor binding, and the like. There will, therefore, be situations where it will be desirable to have constant regions which bind to cells or proteins from a particular species having binding regions for a particular ligand.

B. Relevant Literature

Kwan et al., *J. Exp. Mod.* (1981) 153:1366–1370 and Clarke et al., *Nucl. Acids Res.* (1982) 10:7731–7749 describe V_H and V_κ exons from the mouse phosphocholine-binding antibody-producing S107 myeloma cell line. Oi et al., *Proc. Natl. Acad. Sci. USA* (1983) 80:825–829, report that the mouse light chain gene is not expressed efficiently in a rat myeloma cell.

SUMMARY OF THE INVENTION

Chimeric multi-subunit receptors are provided, where each of the subunits is an expression product of a fused gene. Each fused gene comprises a DNA sequence from one host species encoding the region involved with ligand binding joined to a DNA sequence from a different source, either the same or a different host species, encoding a "constant" region providing a structural framework and biological properties. Introduction of the fused genes into an appropriate eukaryotic host cell under conditions for expression and processing provides for a functional, assembled multi-subunit receptor product.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a schematic diagram of the chimeric mouse; human heavy chain gene vector; and FIG. 1B is the chimeric light chain vector.

FIG. 2 is a schematic diagram of chimeric human IgG anti-DNS expression vectors.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel methods and compositions are provided, for production of polypeptide products having specific binding affinities for a predetermined ligand and predetermined biological, particularly physiological, properties, each of which are not normally associated with the binding region peptide sequences. Particularly, multi-subunit chimeric receptors are provided which result from fused genes having the portion of the polypeptide involved with binding of a predetermined ligand having an amino acid sequence substantially the same (>90% conserved) as an amino acid sequence having the same function from one host, while the portion involved with providing structural stability, as well as other biological functions, being analogously derived from a different host. The resulting composition can be either an inter- or intraspecies chimera. At least two fused genes are involved, which genes are introduced into an appropriate eukaryotic host under conditions for expression and processing, whereby the fused genes are expressed and the resulting subunits bound together, resulting in an assembled chimeric receptor.

The receptors prepared in accordance with the subject invention will be multi-subunit, where the units are held together either by non-covalent binding or a combination of non-covalent and covalent binding, particularly disulfide linkages through cysteine, and having at least one binding site, usually at least two binding sites, and not more than about ten binding sites. Receptors of interest include both B-cell and T-cell receptors, more particularly, immunoglobulins, such as IgM, IgG, IgA, IgD and IgE, as well as the various subtypes of the individual groups. The light chain may be κ or λ. The heavy chains are referred to as μ, γ, α, δ, and ε.

In discussing the two regions of each subunit, the two regions will be referred to as "variable" and "constant" by analogy to immunoglobulins. The variable region is the region involved with ligand binding and, therefore, will vary in conformation and amino acid sequence depending upon the ligand. The region will usually be composed of a plurality of smaller regions (hypervariable or complementary determining regions), involving a region having as its primary function binding to the ligand (V) and a region associated with joining the V region to the constant region, the joining region (J). There may also be a hypervariable region joining the V and J regions, the diversity region (D). These regions are related to gene segments observed in the genes encoding immunoglobulin variable regions.

The constant region will not be associated with ligand binding and will be relatively limited in the variations in its conformation and amino acid sequence within any one species and within any one class, each class generally having from 1 to 4 subclasses. Each constant region is specific for a species. Within the classes there will be allotypes, individual polymorphisms within a class within a species.

The varible region of the immunoglobulins will be derived from a convenient mammalian source, which may be a rodent, e.g., mouse or rat, rabbit, or other vertebrate, mammalian or otherwise, capable of producing immunoglobulins. The constant region of the immunoglobulin, as well as the J chain for IgM and IgA (not the same as the J region of the heavy or light immunoglobulin chain), will be derived from a vertebrate source different from the source of the

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variable region, particularly a mammalian source, more particularly primate or domestic animal, e.g., bovine, porcine, equine, canine, feline, or the like, and particularly, humans. The different source of the constant region can be either from a different species or from the same species as the mammalian source utilized to provide the variable region. Thus, the constant region of the receptor will normally be chosen in accordance with the purpose of the receptor. For example, where the receptor is to be introduced into the host, the constant portion will be selected so as to minimize the immune response of the host to the receptor and to optimize biological efficiency, such as complement fixation or physiological half-life (catabolism). Where the receptor is to bind to particular cell membrane surface receptors, the constant region will be chosen in accordance with the host of the receptor recognition site.

The fused gene derived from the two host sources will be prepared by joining the 5'-end of a sequence encoding the constant region in reading frame to the 3'-end of a sequence encoding the variable region. (In referring to 5' or 3' for a double strand, the direction of transcription is with 5' being upstream from 3'.) With immunoglobulins, two fused genes will be prepared, one for the light chain and one for the heavy chain. With T-cell receptors, the two fused genes will be for each of the two chains involved in the formation of the T-cell receptor. The DNA sequences employed for preparation of the fused gene may be derived from a variety of sources. These sources include genomic DNA, cDNA, synthetic DNA, and combinations thereof. The genomic DNA may or may not include naturally occurring introns.

The DNA obtained from natural sources, namely the genomic DNA or cDNA, may be obtained in a variety of ways. Host cells coding for the desired sequence may be isolated, the genomic DNA may be fragmented, conveniently by one or more restriction endonucleases, and the resulting fragments may be cloned and screened with a probe for the presence of the DNA sequence coding for the polypeptide sequence of interest. For the variable region, the rearranged germline heavy chain DNA will include V, D, and J regions, including the leader sequence, which may be subsequently removed as well as any introns. The rearranged germline light chain coding DNA will include the V and J regions including the leader sequence, as well as any introns which may be subsequently removed. The particular source of the exons defining the domains and the manner of splicing, where introns are present, is not germane to this invention. Once the cloned fragment has been identified which contains the desired DNA sequence, this fragment may be further manipulated to remove superfluous DNA, modify one or both termini, remove all or a portion of intervening sequences (introns), or the like.

In providing a fragment encoding the variable region, it will usually be desirable to include all or a portion of the intron downstream from the J region. Where the intron is retained, it will be necessary that there be functional splice acceptor and donor sequences at the intron termini. The gene sequence between the J (joining region) and the constant region of the fused gene may be primarily the intron sequence associated with (1) the constant region, (2) the J region, or (3) portions of each. The last may be a matter of convenience where there is a convenient restriction site in the introns from the two sources. In some instances, all or a portion of the intron may be modified by deletion, nucleotide substitution(s) or insertion, to enhance ease of manipulation, expression, or the like. When the variable region is chosen to be syngeneic with the host cells employed for expression, all or at least about 80% of the intron sequence can be

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selected from the naturally occurring intron sequence associated with the J region. In some instances it will be necessary to provide adapters to join the intron or truncated intron to the constant region. By cleaving within the intron, the variable region will be separated from its natural constant region.

Alternatively, it may be desirable to have the fused gene free of the intron between the variable and constant regions. Thus, the 3' terminus will be at or in the joining region. Normally all or a portion of the J region will be associated with the host providing the variable region. By restriction enzyme analysis or sequencing of the J region, one can select for a particular site for the 3' terminus of the variable region.

Alternatively, one can use an exonuclease and by employing varying periods of digestion, one can provide for varying 3'-termini, which can then be used for linking to the constant region and selection made for a functional product in a variety of ways. For example, where joining of the variable region to the constant region results in a unique restriction site, the fused DNA fragments may be screened for the presence of the restriction site.

Alternatively, it may be found desirable to include an adapter or linker to join the variable region to the constant region, where the adapter or linker may have the same or substantially the same sequence, usually at least substantially the same sequence, of the DNA sequence of the two fragments adjacent the juncture. The adapter or linker will be selected so as to provide for the two sequences to be in common reading frame. Furthermore, by employing adapters, one could add an additional degree of variability in the binding affinity of the chimeric receptor, by providing for the expression of different amino acids in the J region.

The joining of the various fragments is performed in accordance with conventional techniques, employing blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

For cDNA, the cDNA may be cloned and the resulting clone screened with an appropriate probe for cDNA coding for the desired variable or constant region. Once the desired clone has been isolated, the cDNA may be manipulated in substantially the same manner as the genomic DNA. However, with cDNA there will be no introns or intervening sequences. The cDNA is cleaved at or near the juncture of the variable region with the constant region so that the variable region is separated from the constant region and the desired region retained. Where a convenient restriction site exists, the cDNA may be digested to provide for a fragment having the appropriate terminus. The restriction site may provide a satisfactory site or be extended with an adapter. Alternatively, primer repair may be employed, where for the variable region a complementary sequence to the site of cleavage and successive nucleotides in the 3' direction of the complementary sequence is hybridized to the sense strand of the cDNA and the nonsense strand replicated beginning with the primer and removal of the single-stranded DNA of the sense strand 3' from the primer. The reverse is true for the constant region. Other techniques may also suggest themselves. Once the fragment has been obtained having the predetermined 3' or 5' terminus, as appropriate, it may then be employed for joining to the other region.

Finally, one or both of the regions may be synthesized and cloned for use in preparing the fused gene. For the most part,

the same or substantially the same constant region can be repetitively used, so that a library of constant regions may be established which can be selected for joining to variable regions. Thus, the constant regions would have an appropriate 5' terminus for joining directly or through an adapter to a variable region.

In order for expression of the fused gene, it will be necessary to have transcriptional and translational signals recognized by an appropriate eukaryotic host. For the most part, desirable eukaryotic hosts will be mammalian cells capable of culture in vitro, particularly leukocytes, more particularly myeloma cells, or other transformed or oncogenic lymphocyte, e.g., EBV transformed cells. Alternatively, non-mammalian cells may be employed, such as fungi, e.g., yeast, filamentous fungi, or the like.

The DNA sequence coding for the variable region may be obtained in association with the promoter region from genomic DNA. To the extent that the host cells recognize the transcriptional regulatory and translational initiation signals associated with the variable region, then the region 5' of the variable region coding sequence may be retained with the variable region coding sequence and employed for transcriptional and translational initiation regulation.

The contiguous non-coding region 5' to the variable region will normally include those sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like. Usually the 5'-non-coding sequence will be at least 150 bp, more usually at least 200 bp, usually not exceeding about 2 kbp, more usually not exceeding about 1 kbp.

The non-coding region 3' to the constant region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence coding for the constant region, the transcriptional termination signals may be provided for the fused gene. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted. Conveniently, the non-coding 3' region may be obtained from a non-coding contiguous 3' region of a constant region from the expression host. The 3'-non-coding region may be joined to the constant region by any of the means described previously for manipulation and ligation of DNA fragments. This region could then be used as a building block in preparing the fused gene.

The fused gene for the most part may be depicted by the following formula:

$$\text{TIR-(LS)}_c\text{-V}_f\text{(Vi)}_a\text{-(D)}_b\text{-(Di)}_c\text{-J-(I)}_d\text{-C-TTR}$$
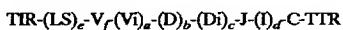
wherein:

TIR intends the transcriptional regulatory and translational initiation region and is generally of at least about 150 bp and not more than about 2 kbp, which may be in whole or in part the sequence naturally joined to the V coding region;

LS refers to a DNA sequence encoding a leader sequence and processing signal functional in the expression host for secretion and processing for removal of the sequence; this leader sequence can contain an intron, as is known in the art to occur;

c is 0 or 1;

V is a segment coding for the variable domain in reading frame with LS, when LS is present;

f is 0 or 1;

D is a segment coding for the diversity domain and is present for the heavy chain (b=1) and is absent for the light chain (b=0);

J is a segment coding for the joining region;

Vi and Di are introns associated with the letter-indicated coding segments having functional donor and acceptor splicing sites;

a, b and c are the same or different and are 0 or 1, wherein when b is 0, c is 0; a, b, and c are all preferably 0;

I is an intron which may be naturally contiguous to the J segment or naturally contiguous to the C domain or a combination of fragments from both, or a fragment thereof, desirably including an enhancer sequence functional in said expression host, or I may be foreign in whole or in part to the J and C segments;

d is 0 or 1 (preferably 0);

C is the constant domain and may code for a μ, γ, δ, α or ϵ chain, preferably μ, γ, or α, usually including at least 80% of the constant region sequence, and may be the same as or a modified naturally occurring allotype or an altered constant region encoding an improved protein sequence; and

TTR is the transcriptional termination region providing for transcriptional termination and polyadenylation which may be naturally associated with C or may be joined to C, being functional in the expression host; usually being at least about 100 bp and may be 1 kbp or more.

Fused genes lacking, or containing modifications in, the hinge region or other immunoglobulin constant region domains can also be prepared, in like manner to the modifications described above, in which case the formula will be as shown above but with the hinge region of the constant chain being absent or modified.

The constructs for each of the different subunits may be joined together to form a single DNA segment or may be maintained as separate segments, by themselves or in conjunction with vectors.

The subunit constructs may be introduced into a cell by transformation in conjunction with a gene allowing for selection where the construct will become integrated into the host genome.

A large number of vectors are available or can be readily prepared which provide for expression in a host, either by maintenance as an extrachromosomal element or by integration into the host genome. For a mammalian host, a wide variety of vectors are known based on viral replication systems, such as Simian virus, bovine papilloma virus, adenovirus and the like. These vectors can be used as expression vectors where transcriptional and translational initiation and termination signals are present and one or more restriction sites are available for insertion of a structural gene. In addition, the vectors normally have one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host; biocide resistance, e.g., resistance to antibiotics, such as G418, or heavy metals, such as copper; or the like. If desired, expression vectors can be prepared by joining the various components, such as the replication system, markers, and transcriptional and translational regulatory initiation and termination signals in conjunction with the fused gene. Frequently, a vector will include a prokaryotic replication system, which allows for cloning, manipulation, purification, and expansion of the desired DNA sequence.

A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression.

Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the fused genes can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical regulation, e.g., metabolite.

Once the vector DNA sequence containing the fused gene has been prepared for expression, the DNA construct may be introduced into an appropriate host. Various techniques may be employed, such as protoplast fusion, calcium phosphate-precipitation, or other conventional technique. After the fusion, the cells are grown in a selective medium or are phenotypically selected leaving only cells transformed with the DNA construct. Expression of the fused gene results in assembly to form the receptor. To date, expression has been accomplished in lymphocytes.

The host cells will for the most part be immortalized cells, particularly myeloma or lymphoma cells. These cells may be grown in an appropriate nutrient medium in culture flasks or injected into a syngeneic host, e.g., mouse or rat, or immunodeficient host or host site, e.g., nude mouse or hamster pouch. Particularly, the cells may be introduced into the abdominal cavity for production of ascites fluid and harvesting of the chimeric receptor. Alternatively, the cells may be injected subcutaneously and the antibodies harvested from the blood of the host. The cells may be used in the same manner as hybridoma cells. See Diamond et al., N. Eng. J. Med. (1981) 3034:1344 and Kennatt, McKearn and Bechtol (eds.), Monoclonal Antibodies: Hybridomas—A New Dimension in Biologic Analysis, Plenum, 1980, which are incorporated herein by reference.

Where a leader is present with a processing signal for secretion and selective cleavage of the leader (signal) sequence, the resulting assembled receptor will be secreted into the nutrient medium of the transformed cells and may be harvested. Where secretion does not occur, after sufficient time for the receptor to be expressed in reasonable amounts, the cells may be killed, lysed, and the receptors isolated and purified. Where transcriptional initiation can be modulated, the cells may be grown to high density under non-permissive conditions, followed by growth under permissive conditions where the receptor is expressed.

The receptors may be naturally glycosylated, unnaturally glycosylated or be free of glycosyl groups, depending on the host, conditions of cellular growth and subsequent treatment. Where a mammalian host cell is employed for expression, usually natural glycosylation will occur. Glycosylation can be prevented by an appropriate inhibitor, e.g., tunicamycin. Alternatively, glycosyl groups may be removed by hydrolysis, e.g., enzymatic hydrolysis using hydrolases. In expression hosts other than mammalian cells, unglycosylated or unnatural glycosylated receptors may be obtained.

The receptor may be isolated and purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like. By employing antibodies specific for the constant region(s), affinity chromatography will allow for concentration and purification of the chimeric receptor.

The chimeric receptors can be used in the same manner as other receptors for binding to specific ligands in diagnostic assays, affinity chromatography or the like. In addition, because a chimeric receptor of substantially reduced immu-

nogenicity can be produced, the chimeric receptors can find use in therapy, for passive immunization, for in vivo imaging, for specific treatment of diseased cells, or the like. For in vivo imaging, the chimeric antibody will normally be conjugated to a radionuclide, e.g., technetium, rhenium, or the like. For biocidal activity, the antibody may be joined to the A-portion of toxins, liposomes containing biocidal reagents, radionuclides, or other biocidal agent. Alternatively, the antibodies can be used in combination with the host immune system, e.g., complement, due to the presence of the native constant region. In vitro, the subject chimeric antibodies can be used in conjunction with complement to remove particular cells from a mixture of cells, where the target cells have a ligand complementary to the binding site of the chimeric antibody.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

Materials and Methods

Chimeric Genes

The cloned S107 variable region (heavy) (V$_H$) and S107 Vκ variable region (light, kappa) genes were obtained from Dr. Matthew Scharff (Dept. of Cell Biology, Albert Einstein College of Medicine, Bronx, N.Y. 10641). The S107 V$_H$ gene was spliced to human IgG1 and IgG2 constant region genes using SalI linkers as shown in FIG. 1A. Both constructs were inserted into the vector pSV2ΔH-gpt (Oi et al., Proc. Natl. Acad. Sci. USA (1983) 80:825–829; Mulligan and Berg, Science (1980) 209:1422–1427). The S107 V$_κ$ gene was spliced to the human κ gene at a unique HindIII site located in the large intron between the J$_κ$ and C$_κ$ exons as shown in FIG. 1B. This chimeric light chain gene construct was inserted into both pSV2ΔH-gpt and pSV2-neo plasmid vectors (Mulligan and Berg, Proc. Natl. Acad. Sci. USA (1981) 78:2072–2076) and pSV184ΔH-neo plasmid vectors (Oi and Morrison, Biotechniques (1986) 4:214–221).

Transfection

Protoplast fusion and calcium-phosphate (CaPO$_4$) precipitation techniques (Oi et al., (1983) supra; Sandri-Goldin et al., Mol. Cell. Biol. (1981) 1:743–752; Chu and Sharp, Gene (1980) 13:197–202) were used to transfect these chimeric immunoglobulin genes into the J558L myeloma cell line (a lambda (λ) light chain producing mouse myeloma cell line) and the non-immunoglobulin-producing An derivative of the P3 myeloma cell line. Mycophenolic acid (Gibco Laboratories, Santa Clara, Calif. 95050) was used for selection of cells transfected with pSV2ΔH-gpt vectors as described previously (Oi et al., (1983) supra; Ochi et al., Proc. Natl. Acad. Sci. USA (1983) 80:6351–6355). G418 (Gibco Laboratories) at 1.0 mg/ml was used for selection of cells transfected with pSV2-neo vectors (Mulligan and Berg, (1980) supra).

When both light and heavy chimeric genes were transfected into the J558L cell line using protoplast fusion techniques, light and heavy chimeric immunoglobulin genes were transfected sequentially using G418 selection for the chimeric light chain gene vector and mycophenolic acid for the chimeric heavy chain gene vector. The protoplast fusion transfection procedure used was as described previously (Oi et al., (1983) supra).

Transfection using the calcium phosphate precipitation procedure was done by mixing 40 μg of both chimeric light and chimeric heavy chain pSV2ΔH-gpt vectors and transfecting a total of 80 μg of plasmid DNA into 5×10^6 cells. Mycophenolic acid was used to select for transformed cell lines as described previously (Oi et al., (1983) supra).

Antigen-binding

Phosphocholine (PC) binding of antibody secreted into the culture supernates of transfected cell lines was analyzed using a solid-phase radioimmunoassay described previously (Oi and Herzenberg, *Mol. Immunol.* (1979) 16:1005–1017). PC-binding antibodies in biosynthetically-labeled culture supernates and cell lysates of transfected cell lines also were analyzed by binding the biosynthetically-labeled antibody to PC-coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) and then eluting the bound antibody with PC-hapten. The bound and eluted antibody was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Biosynthetic-labeling procedures were done as described previously (Oi et al., *J. Exp. Med.* (1980) 151:1260–1274).

Idiotype Analysis

Three hybridoma anti-idiotope antibodies, obtained from Dr. Matthew Scharff (Dept. of Cell Biology, Albert Einstein College of Medicine, Bronx, N.Y.), were used to analyze the variable heavy-variable light (VH-VL) domain structure of the chimeric human anti-PC antibodies. These antibodies, recognizing three independent idiotopes, were used to immunoprecipitate biosynthetically-labeled material eluted with PC from the PC-Sepharose 4B matrix. Immunoprecipitates were analyzed by SDS-PAGE.

Immunoglobulin Chain Composition

Monoclonal anti-human IgG and anti-human κ antibodies (Becton-Dickinson Monoclonal Center, Mt. View, Calif.) were used to immunoprecipitate biosynthetically-labeled chimeric human anti-PC antibodies for analyses using two-dimensional non-equilibrium pH gradient polyacrylamide gel electrophoresis (NEPHGE) (Oi and Herzenberg, (1979) supra). PC-coupled to Sepharose 4B also was used for immunoprecipitations.

Immunoglobulin Heavy Chain Glycosylation

Tunicamycin (Calbiochem-Behring, San Diego, Calif.) was used to inhibit asparagine-linked glycosylation of biosynthetically-labeled antibody from mouse cell lines producing mouse:human chimeric immunoglobulins (Oi et al., (1980) supra). PC-binding antibody from tunicamycin-treated cells was analyzed by SDS-PAGE. Procedures used for tunicamycin treatment were as described previously (Oi et al., (1980) supra).

Chimeric Mouse:Human Antibody Production in Mice

Transformed J558L cells producing chimeric mouse:human antibody were injected subcutaneously into BALB/c mice (10^6 cells/mouse). Sera from tumor-bearing mice were analyzed for human anti-PC antibody by a solid-phase radioimmunoassay described previously (Oi and Herzenberg, (1979) supra) and by immunoelectrophoresis using a polyclonal anti-human antiserum.

Results

Expression of chimeric mouse V:human C region genes in transfected mouse myeloma cells J558L and the non-immunoglobulin-producing P3 myeloma cell lines was obtained. When both light chain and heavy chain chimeric genes were transfected into the same cell, tetrameric (H2L2) antigen-binding antibodies were obtained. Autoradiograms of two-dimensional NEPHGE analyses of the chain composition of biosynthesized and secreted antibody molecules bound and eluted from phosphocholine-Sepharose showed the formation of mixed molecules, including the endogenously produced J558L λ light chain. Each polypeptide chain had the expected charge and relative molecular weight. Identical two-dimensional gel analyses results were obtained with immunoprecipitates with monoclonal anti-human κ and IgG antibodies. Similar results were obtained

from immunoprecipitates of human IgG2(κ) antibodies produced by transfected P3 cell lines. Since the non-immunoglobulin-producing parental P3 cell line does not produce endogenous immunoglobulin polypeptide chains, as expected only the chimeric mouse:human heavy and light chains are seen on the autoradiograms.

Phosphocholine-binding by the chimeric antibody produced in the J558L cell line was the result from specific association of the chimeric immunoglobulin light and heavy polypeptide chains, i.e., the VH and VK domains of the S107 myeloma protein connected to human constant region polypeptides. This was determined by measuring PC-binding by immunoglobulins produced by J558L cells transfected with the mouse:, human chimeric heavy chain gene. PC-binding was never observed from antibody secreted by transfected J558L cells expressing only the chimeric heavy chain and the endogenous J558L λ light chain (data not shown). Chimeric antibodies produced in the transfected non-immunoglobulin-producing P3 cell line also were shown to bind PC-Sepharose. In view of the low binding affinity of the parental mouse S107 antibody, analyses of appropriate polypeptide folding of mouse VH and VK domains in the novel environment of human constant regions polypeptide chains were done by determining the presence of idiotypes known to occur on the parental S107 PC-binding antibody molecule. Three monoclonal anti-idiotope antibodies, each recognizing a distinct epitope on the light and heavy variable region domains and an epitope defined by the presence of both light and heavy variable region domains, were found to react with the mouse:human chimeric anti-PC antibodies. This strongly supports the fact that the mouse S107 antigen-binding domains have folded into their intended structures.

Glycosylation of the mouse:human chimeric antibodies in mouse myeloma cells was analyzed by measuring the relative molecular weight (Mr) of antibodies biosynthesized in the presence and absence of tunicamycin, a known antibiotic inhibitor of asparagine-linked glycosylation. Autoradiograms of SDS-PAGE analysis of the chimeric heavy and light chains produced in mouse myeloma cells in the presence or absence of tunicamycin showed the lower relative Mr of the heavy chain synthesized in the presence of tunicamycin as expected if a single N-linked carbohydrate was absent from the polypeptide chain. From these data it is concluded that the mouse myeloma cell appropriately glycosylates the human heavy chain.

When transfected J558L cells producing the human IgG2 (κ) chimeric anti-PC antibody were grown as a subcutaneous tumor in BALB/c mice, analysis of the sera of these mice showed significant human IgG2(κ) anti-PC binding antibody production by radioimmunoassay. Polyclonal anti-human antiserum demonstrated the presence of significant quantities of human immunoglobulin in the sera. Based on comparison with prior experience with mouse hybridoma-antibody production in mice, the amount of immunoglobulin visualized by immunoelectrophoresis analysis of mice bearing tumors of the transfected J558L cell line was similar to the lower levels of production seen with other mouse hybridoma tumor cell lines.

Analysis showed that fewer than about 10% of the transfected cell lines produced both chimeric heavy and light chain polypeptides. Among transformants generated by protoplast fusion, both gpt and neo biochemical markers were expressed at expected frequencies. However, chimeric light chain expression was infrequent. In co-transfection experiments using the CaPO4 precipitation protocol, the same phenomenon was observed.

Based on prior experience with co-expression of gene products in transformed cell lines, it appears that the appropriate transcriptional or translational controlling elements are absent in either the chimeric light chain gene construct or in the mouse myeloma cell lines used. The mouse V_K gene promoter is coupled to the presumed human intronic DNA sequences that are homologous to the known mouse intronic controlling element (ICE) or immunoglobulin "enhancer" element (Morrison and Oi, *Ann. Rev. Immunol.* (1984) 7:239–256). The chimeric mouse human heavy chain gene is not constructed in this manner and, in contrast, is expressed efficiently. The mouse heavy chain intronic controlling element (ICE) sequences are included and human sequences excluded in this construct. The basis for the low level of expression of the light chain is subject to speculation.

It is evident from the above results that chimeric receptors, as illustrated by immunoglobulins, can be produced where the variable regions may be obtained from one host source and the constant regions obtained from another host source. Where the immunoglobulins are to be used in vivo, this can provide for numerous advantages, such as reduced immunogenicity, a lower catabolism, and the ability to fulfill biological effector functions associated with the constant regions. Furthermore, now that it is shown that chimeric receptors can be produced, there is the opportunity to prepare chimeric receptors with modification of V, J, and D regions so as to modify binding specificity.

EXAMPLE 2

A family of recombinant anti-DNS antibodies was prepared by cloning the mouse V_H gene expressed in the DNS1 (27–44) hybridoma cell line (Dangl et al., *Cytometry* (1982) 2:395) and joining this gene to the already cloned human IgG$_1$, IgG$_2$, IgG$_3$, IgG$_4$, rabbit IgG and mouse IgG$_3$ heavy chain constant region genes. These recombinant genes were inserted into the eukaryotic expression vector, pSV2(delta) H-gpt (Oi et al., *BioTechniques* (1987) 4:214). Each recombinant vector was transfected into a heavy chain loss mutant of the DNS1 hybridoma cell line, 27–44.A5C13, or co-transfected with a DNS-V$_K$ expression vector into the Ig⁻ cell line SP2.0 to generate stable anti-DNS transfectoma cell lines (Morrison and Oi, *Ann. Rev. Immunol.* (1984) 2:239). The expressed V_H and V_K genes from the DNS1 hybridoma (Oi et al., *Nature* (1984) 307:136; Reidler et al., *J. Mol. Biol.* (1982) 158:739) were cloned from phage lambda libraries (Dangl, thesis, Stanford University, Stanford, Calif.) and used to construct the vectors shown in FIG. 2. Transfection by protoplast fusion and selection and screening of transfectomas has been described (Oi et al., *BioTechniques*, supra; Dangl, thesis, Stanford University, Stanford, Calif.). Anti-DNS antibodies were purified from culture supernate by affinity chromatography using a dansyl isomer 2-dimethylaminonaphthyl-5-sulfonamide-3-lysine as absorbant (coupled to AH-Sepharose-4B) and eluant. The affinity of the DNS1 combining site for DNS is 17 nM; the binding affinity of this isomer is lower by a factor of 10^3, making it suitable for use in affinity purification. Removal of bound hapten by dialysis was monitored by fluorescence emission spectroscopy. Antibodies were pure, >95% and free of aggregates as determined by size exclusion chromatography (Dangl, thesis, Stanford University, Stanford, Calif.). The chimeric mouse-human immunoglobulin heavy chain vectors are depicted in FIG. 2. The rabbit IgG and mouse IgG$_3$ heavy chain vectors were similarly constructed.

The nature of the DNS combining site of the genetically engineered antibodies was monitored by measuring the fluorescence emission spectra of bound DNS-lysine. This hapten is a sensitive indicator of the dynamic polarity of its microenvironment. Independently derived mouse IgG anti-DNS antibodies with different combining sites were previously shown to generate different emission spectra (Reidler et al., *J. Mol. Biol.* (1982) 158:739). The absorption and emission spectra of DNS-lysine bound to each genetically engineered antibody was identical to the DNS1 combining site of the parental hybridoma (corrected fluorescence emission spectra of each affinity-purified chimeric antibody were measured using a SIM model 8000 fluorescence spectrophotometer with 340 nm excitation, essentially as described in Reidler, et al., *J. Mol. Biol.* (1982) 158:739). This indicates that each chimeric antibody has a properly folded V_H domain, despite the heterologous junction between the mouse V_H and human or rabbit C_{H1} domains. Stable transfectoma cell lines produced immunoglobulins with heavy chains of appropriate size and charge as determined by two-dimensional sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoglobulins produced by each transfectoma cell line were analyzed by biosynthetic labelling, immunoprecipitation and one- or two-dimensional SDS-PAGE essentially as described by P. P. Jones in *Selected Methods in Cellular Immunology*, B. B. Mishell, S. M. Shiigi, Eds. (W. H. Freeman, San Francisco, 1980), pp. 238. Rabbit anti-human IgG anti-sera was purchased from Cappel. The recombinant heavy chains are glycosylated, a post-translation modification known to affect biosynthetic labeling in the presence or absence of tunicamycin, an antibiotic known to inhibit asparagine-linked glycosylation (Hickman and Kornfeld, *J. Immunol* (1978) 121:990; Nose and Wigzell, *Proc. Natl. Acad. Sci.* (1983) 80:6632; Leatherbarrow et al. *Molec. Immunol.* (1985) 22:407).

This Example demonstrates the ability to prepare inter-species chimeric antibodies from additional species using the previously described techniques as well as the ability to produce intra-species chimerics.

EXAMPLE 3

A series of intra-species hybrid mouse IgG$_1$-IgG$_{2a}$ immunoglobulins all with identical light chains and variable regions were produced. Hybrid heavy chain constant region gene segments were generated by genetic recombination in *E. coli* between plasmids carrying mouse γ1 and γ2a gene segments. Crossovers occurred throughout these segments although the frequency was highest in regions of high nucleotide sequence homology. Eleven hybrid proteins were produced by transfection of a variant hybridoma cell line. Immunoglobulins produced by the transfected cell lines were assembled into H_2L_2 tetramers and properly glycosylated in addition to having identical antigen combining sites specific for the fluorescent hapten ε-dansyl-L-lysine. Protein A binding, used as a probe of Fc structure in these variant antibodies was consistent with the identity of protein A contact residues within the Fc. Novel receptors with new and improved functions were created, demonstrating that improvements over naturally occurring receptors can be made.

Materials and Methods
Generation of hybrid genes

Hybrid C_H gene segments coding for polypeptides with an IgG$_1$ amino-terminus and an IgG$_2$a carboxy-terminus or an IgG$_{2a}$ amino-terminus and IgG$_1$ carboxy-terminus were generated by adaptation of the system described by Schneider et al., *Proc. Natl. Acad. Sci.* (1981)

78:2169–2173. Plasmid pHGX1 was constructed by digesting pBN2 (Nichols and Yanofsky, *Proc. Natl. Acad. Sci.* (1979) 76:5244–5248) with HindIII and PvuII, filling in the overhanging HindIII ends with T_4 DNA polymerase, ligating XbaI linkers to the flush ends, and recircularizing the plasmid. Plasmids pHGX1C$_{\gamma 2a}$A and pHGX1C$_{\gamma 2a}$B were generated by digesting pγ2a.9 with EcoRI and HindIII, filling in the overhanging ends, ligating XbaI linkers to the blunt ends, and inserting the C$_{\gamma 2a}$-containing fragment into XbaI-digested pHGX1. Plasmids pHGX1C$_{\gamma 2a}$A and pHGX1C$_{\gamma 2a}$B differ only in the orientation of the inserted fragment. Plasmid pHGX2 was constructed by digesting pWS1 (Schneider et al., *Proc. Natl. Acad. Sci.* (1981) 78:2169–2173) with HpaI and SalI, filling in the overhanging SalI end, ligating XbaI linkers to the flush ends, and recircularizing the plasmid. Plasmids pHGX2C$_{\gamma 1}$A and pHGX2C$_{\gamma 1}$B were generated by digesting P$_{\gamma 1}$ with KpnI, and ligating the C$_{\gamma 2a}$-containing fragment to XbaI-digested pHGX2. Plasmids pHGX2C$_{\gamma 1}$A and pHGX2C$_{\gamma 1}$B differ only in the orientation of the inserted fragment.

E. coli strain W3110 trpR ΔtrpEA2 tnaA2 rna-19 was transformed with both pHGX1C$_{\gamma 2a}$A and pHGX1C$_{\gamma 1}$A or pHGX1C$_{\gamma 2a}$B and pHGX1C$_{\gamma 1}$B to chloramohenicol resistance (Cm^r) and ampicillin resistance (Amp^r). Single colonies were transferred to L broth containing chloramphenicol (20 mg/l) and ampicillin (100 mg/l) and grown overnight. Cells were collected by centrifugation, washed with Vogel-Bonner minimal medium (Vogel and Bonner, *J. Biol. Chem.* (1956) 218:97–106), and plated on minimal plates supplemented with glucose (0.4%), acid-hydrolyzed casein (0.5%), indole (10 mg/l) and chloramphenicol. Individual colonies were transferred to identical liquid medium and grown overnight. Plasmid DNA was extracted from these cultures, and the monomeric double crossover plasmids isolated by size fractionation using agarose gel electrophoresis. DNA from monomeric plasmid fractions was used to transform *E. coli* W3110 trpR ΔtrpEA2 tnaA2 rna-19 to Trp$^+$ and Cm^r. Transformants were screened for ampicillin sensitivity (Amp^s) by replica plating.

The crossover site generating each hybrid was located by restriction enzyme analysis. Precise crossover junctions were identified by DNA sequencing by the method of Maxam and Gilbert/*Methods Enzymol.* (1980) 65:499–560 or Sanger and Coulson, *Proc. Natl. Acad. Sci.* (1977) 74:5463–5467 using M13mp9 (Messing and Vieira, *Gene* (1982) 19:269–276) to generate single-stranded DNA template.

An additional variant heavy chain gene, one lacking the hinge region exon, also was constructed. The XbaI ends of fragments containing C$_H$ gene segments were filled in and converted to SalI ends with linkers. These fragments were ligated into the SalI site of pMLSVgpt DNS-V$_H$. A γ$_{2a}$ heavy chain constant region with a hinge exon deletion was constructed by removing a StuI-SmaI fragment from an expression vector carrying the γ$_{2a}$ C$_H$ coding region. This plasmid is designated pMLSVgpt DNS-V$_H$C$_{\gamma 2a}$Δ hinge.

Transfection of Hybrid Heavy Chain Genes

Plasmids designed to express anti-DNS hybrid heavy chain genes were constructed as described above. The pMLSVgpt DNA-V$_H$ contains the *E. coli* xanthine-guanine phosphoribosyltransferase gene (gpt) which is used to biochemically-select transfected mammalian cells (Mulligan and Berg, *Proc. Natl. Acad. Sci.* (1981) 78:2072–2076). The heavy chain variable region (V$_H$) gene segment from the anti-DNS hybridoma 27–44 (Dangl et al., *Cytometry* (1982) 2:395–401) is located upstream of the C$_H$ gene insertion site. Hence ligation of each recombinant

heavy chain constant region gene segment into pMLSVgpt DNA-V$_H$ in the proper orientation generates a gene encoding the corresponding variant anti-DNS heavy chain polypeptide. Hybrid anti-DNS antibody-producing cell lines were generated by transfecting these recombinant vectors into a heavy-chain-loss variant of the 27–44 hybridoma cell line, designated 27–44 A5C13 (which was provided by Dr. D. Parks of Stanford University). Other hybridoma cell lines lacking production of the heavy chain could also be used.

Expression vectors were transfected into 27–44 A5C13 using the following protoplast fusion procedure. *E. coli* strain HB101 carrying the appropriate plasmid was grown in L broth to an OD$_{600}$ of 0.6–0.7. Plasmid copy number was amplified by addition of chloramphenicol (170 mg/l) and incubating cultures overnight. Cells from 25 ml of culture were harvested by centrifugation and gently resuspended in 1.25 ml of ice cold 20% sucrose, 50 mM Tris-HCl pH 8.0. Freshly prepared lysozyme solution (0.25 ml at 5 mg/ml in 0.25M Tris-HCl pH 8.0) was added and the suspension incubated for 6 minutes on ice. A 0.5 ml aliquot of 0.25 mM EDTA pH 8.0 was added and the mixture was incubated for 5 minutes on ice. Following addition of 0.5 ml of 50 mM Tris-HCl pH 8.0, the cells were incubated for 10 minutes at 37° C. The bacteria were then diluted with 10 ml of RPMI 1640 medium supplemented to 10% sucrose and 10 mM MgCl$_2$ and warmed to 37° C. After incubation for 10 minutes at room temperature, the protoplasts were used for fusion.

The 27–44 A5C13 cell line was grown to a density of 0.3 to 1×10^6 cells/ml in RPMI 1640 medium containing 1.0-mM sodium pyruvate, 2.0 mM L-glutamine, 50 mM β-mercaptoethanol, 10% fetal calf serum (FCS), and 1% NuSerum™ (Collaborative Research, Lexington, Mass.). Cells (2×10^7) were harvested by centrifugation and resuspended in 1 ml of RPMI 1640 medium with supplements. After addition of 3 ml of protoplast suspension, the cells were pelleted by centrifugation. The pellet was resuspended by slowly adding 1 ml of 45% polyethylene glycol (PEG) in RPMI 1640 medium without supplements (warmed to 37° C.) while mixing gently for 1 to 2 minutes. Nine milliliters of 37° C/RPMI medium without supplements were added slowly to dilute the PEG solution. The suspension was centrifuged and the pellet resuspended in 12 ml of RPMI 1640 medium. Cells were then dispensed into a 24-well plate in 0.5 ml aliquots. The following day, 1 ml of RPMI 1640 medium was added to each well. Two days after fusion, transfected cell lines were selected in medium containing 1 mg/l mycophenolic acid, 15 mg/l hypoxanthine, and 200 mg/l xanthine. Cultures were fed with 1 ml of selection medium as needed.

Protein Characterization

Antibodies from transfected cell lines were biosynthetically-labeled with [^{35}S]-methionine in the absence and presence of tunicamycin. Antibodies were immunoprecipitated with rabbit anti-mouse immunoglobulin antisera and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional non-equilibrium pH gradient gel electrophoresis, and two-dimensional non-reducing-reducing polyacrylamide gel electrophoresis (Goding, *Handbook of Experimental Immunology, Vol.* 1. Blackwell Scientific Publications, Oxford (1986) 20:1–20.33). Fluorescence emission spectra of hybrid anti-DNS antibodies bound to ε-dansyl-L-lysine were measured using 340 nm excitation on a SLM model 8000 fluorescence spectrophotometer.

Production and Purification of Genetically Engineered Antibodies

Hybrid anti-DNS antibodies were purified from sera of tumor-bearing mice or from culture supernates of transfected cell lines grown in serum-free medium (Hana Biologicals, Berkeley, Calif.). Hybridoma cell lines 27–44 and 27–13 were used to produce parental IgG₁ and IgG₂a anti-DNS antibodies (Dangl et al., *Cytometry* (1982) 2:395–401).

Anti-DNS antibodies were purified by affinity chromatography using a DNS analog (2-dimethylaminonaphthyl-5-sulfonyl chloride; Molecular Probes, Eugene, Oreg.) coupled to AH-Sepharose 4B (Pharmacia, Piscataway, N.J.). N-(5-Carboxypentyl)-2-dimethylaminonaphthyl-5-sulfonamide was used to elute bound antibody.

Protein A Binding

Binding of each hybrid anti-DNS antibody to *Staphylococcus aureus* protein A was measured at pH 7.0. Purified protein A (Pharmacia) and a two-fold molar excess of IgG were incubated in 150 mM sodium phosphate pH 7.0 for 20 minutes at room temperature. IgG-protein A complexes were analyzed using Superose 6 column chromatography (Pharmacia).

Results

Generation of Hybrid C_H Genes

Genetic recombination between pHGX1C$_{\gamma 2a}$A and pHGX1C$_{\gamma 1}$A or pHGX1C$_{\gamma 2a}$B and pHGX1C$_{\gamma 2a}$B yielded TRP⁺ colonies at frequencies similar to that reported previously (Schneider et al., supra). The Trp⁺ phenotype results from a single crossover between trpB segments in the two parental plasmids to generate an intact, functional gene. The single crossover produces a dimeric plasmid, containing the entire genetic material of both starting plasmids. The dimeric trpB⁺ Cmʳ Ampʳ plasmid is expected to sustain a second recombinational event in any region of homology. A second crossover, between $C_\gamma 1$ and $C_\gamma 2a$ gene segments, yields a monomeric trpB⁺ Cmʳ Ampˢ plasmid with a hybrid C_H segment. Transformation of *E. coli* W3110 trpR ΔtrpEA2 tnaA2 rna-19 with size-fractionated monomeric plasmid DNA from trpB⁺ cells, followed by selection for Trp⁺ and Cmʳ, yielded only monomeric trpB⁺ Cmʳ Ampˢ plasmids that always carried hybrid C_H gene segments.

Seventy independently derived hybrid C_H gene segments were recovered. Ten of the most diverse recombinant immunoglobulin heavy chain constant region gene segments were selected for further study. Six have an IgG₁ amino-terminus and an IgG₂a carboxy-terminus (designated $\gamma_1\gamma_{2a}^{-2 \ through}$). Five of these six recombinant molecules were generated by crossovers in the C_H2 exon; the sixth was generated by a crossover in the intron between the C_H1 and hinge exons. Two recombinants (designated $\gamma_{2a}\gamma_1^{-1 \ and \ -2}$) are IgG₂a-IgG₁ hybrids; one generated by a crossover in the C_H2 exon and the other by a crossover in the intron between the C_H1 and hinge exons. Two other recombinants ($\gamma_1\gamma_{2a}^{-1 \ and \ -8}$) were included for study as controls. They encode parental γ_1 and γ_{2a} heavy chain polypeptides and arose from crossovers in DNA sequences flanking immunoglobulin coding regions.

Precise crossover points were determined by DNA sequencing. The amino acid sequences of the parental IgG₁ and IgG₂a polypeptides as well as the deduced sequence junctions of the ten recombinant heavy chains were determined. Nine of the ten hybrid genetic segments appear to have been generated by single crossover events, while $\gamma_1\gamma_{2a}^{-1}$ appears to have arisen by a triple exchange within the intron between C_H1 and hinge exons. The length of nucleotide sequence identity between $C_{\gamma 1}$ and $C_{\gamma 2a}$ gene segments within which exchanges occurred varied from 5 to 21 base pairs.

Expression of Recombinant Immunoglobulins Genes

All eleven heavy chain genes are expressed when transfected into the 27–44 A5C13 cell line. SDS-PAGE analyses show that each cell line synthesizes and secretes, in addition to light chain (L), an immunoglobulin heavy chain (H) polypeptide of the appropriate relative molecular weight (Mr). Two-dimensional nonequilibrium pH gradient gel electrophoresis analysis confirmed the identity of the endogenous light chain produced by each transfected cell line, as well as the expected charge (deduced from the amino acid sequence) for each recombinant heavy chain polypeptide. Each recombinant antibody, when synthesized in the presence of tunicamycin showed an altered mobility upon reducing SDS-PAGE relative to each antibody synthesized in the absence of tumicamycin indicating that each heavy chain is glycosylated.

The DNS fluorescence emission spectrum of all eleven recombinant antibodies are identical to the hybridoma IgG₁ and IgG₂a anti-DNS antibody fluorescence emission spectra. This is an accurate and sensitive means of identifying the antigen-combining sites of anti-DNS antibodies (Reidler et al., *J. Mol. Biol.* (1982) 158:739–746, Oi et al., *Nature* (1984) 307:136–140) and demonstrates that the original DNS combining sites have been re-created in the hybrid antibodies.

Interchain Disulfide Bridges

Each genetically engineered anti-DNS antibody was assembled into H₂L₂ tetramers as determined using Superose 6 column chromatography. The presence of interchain disulfide bridges was ascertained using non-reducing SDS-PAGE. Eight of ten hybrid immunoglobulins have mobilities characteristic of H₂L₂ molecules, having both H-L and H-H disulfide bridges. Analysis of the hingeless IgG₂a antibody shows H-L molecules and the absence of H₂L₂ tetramers. This was expected since the cysteines forming the H-H disulfide bridges are located in the hinge region. The two remaining hybrid immunoglobulins have unusual disulfide bridges.

Protein A Binding

The protein A binding of each of the eleven variant antibodies was assessed and used as a probe of Fc structural integrity. Table 1 summarizes the results of this analysis.

TABLE 1

Amino acid residues at positions of protein A contact and protein A binding of hybrid and hinge deleted antibodies.

Antibody	252	253	254	308	309	310	311	312	433	434	435	436	Protein A Binding
						Contact Residues							
$\gamma_1\gamma_{2a}^{-1}$(IgG2a)	Met	Ile	Ser	Ile	Gln	His	Gln	Asp	His	Asn	His	His	+
$\gamma_1\gamma_{2a}^{-2}$	–	–	–	–	–	–	–	–	–	–	–	–	+

TABLE 1-continued

	Amino acid residues at positions of protein A contact and protein A binding of hybrid and hinge deleted antibodies.												
	Contact Residues												Protein A
Antibody	252	253	254	308	309	310	311	312	433	434	435	436	Binding
$\gamma_1\gamma_{2a}^{-3}$	–	–	–	–	–	–	–	–	–	–	–	–	+
$IgG_{2a}\Delta hinge$	–	–	–	–	–	–	–	–	–	–	–	–	+
$\gamma_1\gamma_{2a}^{-8}(IgG1)$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_{2a}^{-6}$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_{2a}^{-7}$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_1^{-1}$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_1^{-2}$	Thr	–	Thr	–	Met	–	–	–	–	–	–	–	–
$\gamma_1\gamma_{2a}^{-4}$	Thr	–	Thr	–	–	–	–	–	–	–	–	–	–
$\gamma_1\gamma_{2a}^{-5}$	Thr	–	Thr	–	–	–	–	–	–	–	–	–	–

Contact residues in $\gamma_1\gamma_{2a}$ are given. EU numbering is used.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method for producing a functional immunoglobulin comprising a heavy chain and a light chain, which comprises the steps of:

(a) transfecting a transformed mammalian lymphocytic cell with a first DNA molecule coding for a first chain of the immunoglobulin;

(b) transfecting the cell with a second DNA molecule, said second DNA molecule coding for a second chain of the immunoglobulin, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain; and

(c) maintaining the cell in a nutrient medium, so that the cell expresses the first and second DNA molecules and the resultant chains are intracellularly assembled together to form the immunoglobulin which is then secreted in a form capable of specifically binding to antigen wherein prior to step (a) the cell does not express a functional immunoglobulin capable of specifically binding antigen.

2. A method as recited in claim 1 wherein the cell is transfected via protoplast fusion.

3. A method as recited in claim 1 wherein the cell is transfected via calcium phosphate precipitation.

4. A method as recited in claim 1 wherein the cell is a myeloma cell.

5. A method as recited in claim 4 wherein the cell is a murine myeloma cell.

6. A method as recited in claim 1 wherein the cell does not endogenously produce any immunoglobulin chains.

7. A method as recited in claim 6 wherein the cell is a murine P_3 cell.

8. A method as recited in claim 1 wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

9. A method as recited in claim 8 wherein the cell is a murine J558L cell.

10. A method as recited in claim 1 wherein the immunoglobulin comprises the variable region found in a first mammalian species and comprises the constant region found in a second mammalian species, said second mammalian species being other than the first mammalian species.

11. A method for producing a functional immunoglobulin comprising a heavy chain and a light chain, which comprises the steps of:

(a) transfecting a transformed mammalian lymphocytic cell with a plasmid comprising a first DNA molecule coding for a first chain of the immunoglobulin and a second DNA molecule coding for a second chain of the immunoglobulin, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain; and

(b) maintaining the cell in a nutrient medium so that the cell expresses said first DNA molecule and said second DNA molecule and the resultant chains are intracellularly assembled together to form the immunoglobulin which is then secreted in a form capable of specifically binding to antigen wherein prior to step (a) the cell does not express a functional immunoglobulin capable of specifically binding antigen.

12. A method as recited in claim 11 wherein the cell is transfected via protoplast fusion.

13. A method as recited in claim 11 wherein the cell is transfected via calcium phosphate precipitation.

14. A method as recited in claim 11 wherein the cell is a myeloma cell.

15. A method as recited in claim 14 wherein the cell is a murine myeloma cell.

16. A method as recited in claim 11 wherein the cell does not endogenously produce any immunoglobulin chains.

17. A method as recited in claim 16 wherein the cell is a murine P_3 cell.

18. A method as recited in claim 11 wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, which endogenously-produced heavy chain is not secreted in a form capable of specifically binding to antigen, but not both.

19. A method as recited in claim 18 wherein the cell is a murine J558L cell.

20. A method as recited in claim 11 wherein the immunoglobulin comprises the variable region found in a first mammalian species and comprises the constant region found

in a second mammalian species, said second mammalian species being other than the first mammalian species.

21. A method for producing a functional immunoglobulin comprising a heavy chain and a light chain which comprises the steps of:

(a) maintaining in a nutrient medium a transformed mammalian lymphocytic cell, said cell having been transfected with a first DNA molecule coding for a first chain of the immunoglobulin and a second DNA molecule coding for a second chain of the immunoglobulin, said second chain being a chain other than the first chain and said first and second chains being either the heavy chain or the light chain;

(b) expressing from said cell the heavy chain and the light chain functionally assembled together to form said immunoglobulin which is then secreted in a form capable of binding antigen; and

(c) recovering said immunoglobulin wherein prior to being transfected, the cell does not express a functional immunoglobulin capable of specifically binding antigen.

22. A method as recited in claim 21 wherein the cell is transfected via protoplast fusion.

23. A method as recited in claim 21 wherein the cell is transfected via calcium phosphate precipitation.

24. A method as recited in claim 21 wherein the cell is a myeloma cell.

25. A method as recited in claim 24 wherein the cell is a murine myeloma cell.

26. A method as recited in claim 21 wherein the cell does not endogenously produce any immunoglobulin chains.

27. A method as recited in claim 26 wherein the cell is a murine (P_3) cell.

28. A method as recited in claim 21 wherein prior to being transfected the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

29. A method as recited in claim 28 wherein the cell is a murine J558L cell.

30. A method as recited in claim 21 wherein the immunoglobulin comprises the variable region found in a first mammalian source and comprises the constant region found in a second mammalian species, said second mammalian species being other than the first mammalian species.

31. A method for producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the method comprises the steps of:

(a) transfecting a transformed mammalian lymphocytic cell with a first DNA molecule coding for the first chain of the protein;

(b) transfecting the cell with a second DNA molecule, said second DNA molecule coding for the second chain of the protein; and

(c) maintaining the cell in a nutrient medium, so that the cell expresses the first and second DNA molecules and the resultant chains are intracellularly assembled together to form the protein which is then secreted in a form capable of specifically binding to antigen

wherein prior to step (a) the cell does not express a functional antigen-binding protein.

32. A method as recited in claim 31 wherein prior to step (a) the cell does not endogenously produce any immunoglobulin chains.

33. A method as recited in claim 31 wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

34. A method as recited in claim 31 wherein the first chain comprises a constant region.

35. A method as recited in claim 31 wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

36. A method as recited in claim 34 wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

37. A method for producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the method comprises the steps of:

(a) transfecting a transformed mammalian lymphocytic cell with a plasmid comprising a first DNA molecule coding for the first chain of the protein and a second DNA molecule coding for the second chain of the protein; and

(b) maintaining the cell in a nutrient medium so that the cell expresses said first DNA molecule and said second DNA molecule and the resultant chains are intracellularly assembled together to form the protein which is then secreted in a form capable of specifically binding to antigen

wherein prior to step (a) the cell does not express a functional immunoglobulin capable of specifically binding antigen.

38. A method as recited in claim 37 wherein prior to step (a) the cell does not endogenously produce any immunoglobulin chains.

39. A method as recited in claim 37 wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

40. A method as recited in claim 37 wherein the first chain comprises a constant region.

41. A method as recited in claim 39 wherein the antigen-binding protein comprises the heavy and light chain variable domains are from a first mammalian species and comprises the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

42. A method as recited in claim 40 wherein the antigen-binding protein comprises the heavy and light chain variable domains are from a first mammalian species and comprises the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

43. A method for producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

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wherein the method comprises the steps of:

(a) maintaining in a nutrient medium a transformed mammalian lymphocytic cell, said cell having been transfected with a first DNA molecule coding for the first chain of the protein and a second DNA molecule coding for the second chain of the protein;

(b) expressing from said cell the first and second chains functionally assembled together to form said protein which is then secreted in a form capable of binding antigen; and

(c) recovering said antigen-binding protein, wherein prior to being transfected, the cell does not express a functional immunoglobulin capable of specifically binding antigen.

44. A method as recited in claim 43 wherein prior to step (a) the cell does not endogenously produce any immunoglobulin chains.

45. A method as recited in claim 43 wherein prior to step (a) the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

46. A method as recited in claim 43 wherein the first chain comprises a constant region.

47. A method as recited in claim 43 wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

48. A method as recited in claim 46 wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

49. A transformed mammalian lymphocytic cell producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the transformed mammalian lymphocytic cell comprises:

(a) a first exogenous DNA molecule coding for the first chain of the protein; and

(b) a second exogenous DNA molecule, said second DNA molecule coding for the second chain of the protein;

wherein without the exogenous DNA molecules the cell does not express a functional antigen-binding protein.

50. A transformed mammalian lymphocytic cell as recited in claim 49 wherein without the exogenous DNA molecules the cell does not endogenously produce any immunoglobulin chains.

51. A transformed mammalian lymphocytic cell as recited in claim 49 wherein without the exogenous DNA molecules the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

52. A transformed mammalian lymphocytic cell as recited in claim 49 wherein the first chain comprises a constant region.

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53. A transformed mammalian lymphocytic cell as recited in claim 49 wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

54. A transformed mammalian lymphocytic cell as recited in claim 52 wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

55. A transformed mammalian lymphocytic cell producing a functional antigen-binding protein comprising

i) a first chain comprising an immunoglobulin heavy chain variable domain and an immunoglobulin heavy chain constant domain and

ii) a second chain comprising an immunoglobulin light chain variable domain and an immunoglobulin light chain constant domain,

wherein the transformed mammalian lymphocytic cell comprises:

a plasmid comprising a first exogenous DNA molecule coding for the first chain of the protein and a second exogenous DNA molecule coding for the second chain of the protein; and

wherein without the exogenous DNA molecules the cell does not express a functional antigen-binding protein.

56. A transformed mammalian lymphocytic cell as recited in claim 55 wherein without the exogenous DNA molecules the cell does not endogenously produce any immunoglobulin chains.

57. A transformed mammalian lymphocytic cell as recited in claim 55 wherein without the exogenous DNA molecules the cell endogenously produces an immunoglobulin light chain or an immunoglobulin heavy chain, but not both.

58. A transformed mammalian lymphocytic cell as recited in claim 55 wherein the first chain comprises a constant region.

59. A transformed mammalian lymphocytic cell as recited in claim 55 wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant domains are from a second mammalian species, said second mammalian species being other than the first mammalian species.

60. A transformed mammalian lymphocytic cell as recited in claim 58 wherein the heavy and light chain variable domains are from a first mammalian species and the heavy and light chain constant regions are from a second mammalian species, said second mammalian species being other than the first mammalian species.

61. A transformed mammalian lymphocytic cell produced by steps (a) and (b) of claim 31.

62. A transformed mammalian lymphocytic cell produced by step (a) of claim 37.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO : 5,807,715

DATED   : September 15, 1998

INVENTOR(S): S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

In the Title [54], delete "LYMPHOCYTE" and replace with --LYMPHOCYTIC--; after "IMMUNOGLOBULIN", add --AND FRAGMENTS--.

In the Inventors [75], delete "Scarsdale, N.Y." and replace with --Los Angeles--; delete "Menlo Park" and replace with --Mountain View--; delete "both" and replace with --all--.

In the Assignee [73], delete "Assignee" and replace with --Assignees--; after "Calif." add --, and The Trustees of Columbia University, New York, N.Y.--.

In References Cited [56], after "Seno et al 1983 Nucleic", delete "Acid" and replace with --Acids--; after "Research 11(3)", delete ";" and replace with --:--.

In References Cited [56], after "Dolby et al 1980 PNAS 77(10)" add --:--.

Column 1, line 2, delete "LYMPHOCYTE" and replace with --LYMPHOCYTIC--.

Column 1, line 5, after "IMMUNOGLOBULIN" add --AND FRAGMENTS--.

Column 1, line 16, add the paragraph --The work described herein was supported in part by grants from the National Institutes of Health (NIH), including AI-00408, AI-08917, CA-04681, and CA-16858. The United States Government has certain rights in the invention.--

Column 1, line 19, delete "1.".

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DATED : September 15, 1998

INVENTOR(S): S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 1, line 41, delete "2.".

Column 1, line 61, after "functional", add --,--; after "assembled", add --,--.

Column 1, line 66, delete "mouse-" and replace with --mouse:--.

Column 1, line 67, delete ":".

Column 3, line 26, delete "prepar" and replace with --prepara--.

Column 3, line 32, delete "CDNA" and replace with --cDNA--.

Column 4, line 45, delete "CDNA" and replace with --cDNA--.

Column 4, line 52, delete "CDNA" and replace with --cDNA--.

Column 4, line 59, delete "CDNA" and replace with --cDNA--.

Column 4, line 65, delete "Joining" and replace with --joining--.

Column 8, line 2, after "immunization", add --,--.

Column 8, line 21, delete "VH" and replace with $--V_H--$.

Column 8, line 22, delete "VK" and replace with $--V_\kappa--$ (Greek letter kappa subscripted).

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DATED   : September 15, 1998

INVENTOR(S): S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 8, line 24, delete "VH" and replace with --V_H--.

Column 8, line 29, delete "Vκ" and replace with --$V_κ$-- (Greek letter kappa subscripted).

Column 8, line 31, delete "Jκ" and replace with --$J_κ$-- (Greek letter kappa subscripted); delete "Cκ" and replace with --$C_κ$-- (Greek letter kappa subscripted).

Column 9, line 9, delete "Five" and replace with --Fine--; delete "Chamicals" and replace with --Chemicals--.

Column 9, line 19, delete "VH-VL" and replace with --V_H-V_L--.

Column 9, line 57, delete "H2L2" and replace with --H_2L_2--.

Column 9, line 67, delete "K" and replace with --κ--; delete "antiodies" and replace with --antibodies--.

Column 10, line 5, after "expected" add --,--.

Column 10, line 10, delete "VH" and replace with --V_H--; delete "Vκ" and replace with --$V_κ$-- (Greek letter kappa subscripted).

Column 10, line 14, delete "mouse: human" and replace with --mouse:human--.

Column 10, line 22, delete "VH" and replace with --V_H--; delete "Vκ" and replace with --$V_κ$-- (Greek letter kappa subscripted).

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DATED   : September 15, 1998

INVENTOR(S): S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 10, line 36, delete "Mr" and replace with --M_r--.

Column 10, line 42, delete "Mr" and replace with --M_r--.

Column 10, line 54, delete "hybri-doma-" and replace with --hybridoma--.

Column 11, line 5, delete "Vκ" and replace with --V_κ -- (Greek letter kappa subscripted).

Column 11, line 10, delete "mouse: human" and replace with --mouse:human--.

Column 11, line 21, delete "use" and replace with --used--.

Column 11, line 35, delete "IgG_1, IgG_2, IgG_3, IgG_4, rabbit IgG and mouse IgG_3" and replace with --IgG1, IgG2, IgG3, IgG4, rabbit IgG and mouse IgG3--.

Column 11, line 37, delete "(delta)" and replace with --Δ-- (Greek letter capital delta).

Column 11, line 41, delete "V_κ" and replace with --V_κ-- (Greek letter kappa subscripted).

Column 11, line 43, after "Ann" add --.--

Column 11, line 44, delete "V_κ" and replace with --V_κ-- (Greek letter kappa subscripted).

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PATENT NO. 5,807,715

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UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO   : 5,807,715

DATED   : September 15, 1998

INVENTOR(S) : S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 11, line 60, delete "pure, >95%" and replace with -- pure (>95%) --.

Column 11, line 63, delete "mouse-human" and replace with -- mouse:human --.

Column 11, line 64, delete "IgG₃" and replace with --IgG3--.

Column 12, line 3, delete "IgG₁" and replace with --IgG1--.

Column 12, line 10, delete "affinity purified" and replace with --affinity-purified--.

Column 12, line 22, delete "labelling" and replace with -- labeling --.

Column 12, line 26, delete "pp." and replace with --p.--

Column 12, line 31, after "Kornfeld" add --,--

Column 12, line 32, delete "Wigzell *Proc*," and replace with --Wigzell, *Proc.*--.

Column 12, line 33, after "et al." add --,--.

Column 12, line 42, delete "IgG₁-IgG₂ₐ" and replace with -- IgG1-IgG2a --.

Column 12, line 64, delete "IgG₁" and replace with --IgG1--; delete "IgG₂a" and replace with --IgG2a--.

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INVENTOR(S) : S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 12, line 65, delete "IgG_{2a}" and replace with --IgG2a--; delete "IgG_1" and replace with --IgG1--.

Column 13, line 4, delete "T_4" and replace with --T4--

Column 13, line 43, after "Gilbert" add --,--

Column 13, line 65, delete "Dangi" and replace with --Dangl--

Column 14, line 24, delete "bateria" and replace with --bacteria--.

Column 14, line 41, delete "C." and replace with --C--.

Column 14, line 53, delete "labelled" and replace with --labeled--.

Column 15, line 5, delete "IgG_1" and replace with --IgG1--; delete "IgG_2a" and replace with --IgG2a--.

Column 15, line 25, delete "$pHGX1C_{v_2}aB$" and replace with --$pHGX1C_{v_1}B$--.

Column 15, line 27, delete "Trp^+" and replace with --TRP^+--.

Column 15, line 39, delete "Trp^+" and replace with --TRP^+--.

Column 15, line 44, delete "IgG_1" and replace with --IgG1--

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PATENT NO : 5,807,715

DATED : September 15, 1998

INVENTOR(S) : S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 15, line 45, delete "IgG$_{2a}$" and replace with --IgG2a--

Column 15, line 49, delete "IgG$_{2a}$" and replace with --IgG2a--

Column 15, line 50, delete "IgG$_1$" and replace with --IgG1--

Column 15, line 46, delete "-7" and replace with --$^{-7}$--.

Column 16, line 2, delete "IgG$_1$" and replace with --IgG1--

Column 16, line 3, delete "IgG$_{2a}$" and replace with --IgG2a--

Column 16, line 18, delete "Mr" and replace with --M$_r$--

Column 16, line 29, delete "are" and replace with --is--; delete "IgG$_1$" and replace with --IgG1--.

Column 16, line 30, delete "IgG$_{2a}$" and replace with --IgG2a--

Column 16, line 44, delete "IgG$_{2a}$" and replace with --IgG2a--

Column 17, line 63 (claim 7), delete "P$_3$" and replace with --P3--.

Column 18, line 57 (claim 17), delete "P$_3$" and replace with --P3--.

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DATED   : September 15, 1998

INVENTOR(S) : S.L. Morrison, Herzenberg, L.A., and Oi, V.T.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 19, line 33 (claim 27), delete "P_3" and replace with --P3--.

Column 20, line 50 (claim 41), delete "39" and replace with --37--.

Column 20, line 52 (claim 41), delete "are".

Column 20, line 53 (claim 41), delete "are".

Column 20, line 58 (claim 42), delete "are".

Column 20, line 59 (claim 42), delete "are".

Column 21, line 13, shift line beginning with "express" to left margin.

Column 21, line 14, shift line beginning with "specifically" to left margin.

Column 21, line 29 (claim 48), delete "arc" and replace with --are--.

Column 22, line 43 (claim 59), delete "arc" and replace with --are--.

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